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Ross v. [illegible] No. 80-4637
14th Judicial District Court
Calcasieu Parish, Louisiana

July 31, 1978

TO: Vinyl Chloride Technical Panel
Vinyl Chloride Research Coordinators
Vinyl Chloride Medical Subcommittee

Gentlemen:

As reported in Section 6.0 of the Record of the University of Louisville Meeting on June 14, 1978, Drs. Tamburro and O'Connell promised to send the Project Manager complete abstracts of all research reviews presented on all component studies. We are pleased to report that they have exceeded their promise by sending us complete transcripts of the actual research reviews covering Dr. Tamburro's "Overview of the University of Louisville Multi-Disciplinary Approach to the Study of Chemical Carcinogenesis" and the reports on the component research projects as well. Included in the enclosed collection of papers are copies of all slides which were presented during the June 14th meeting.

We trust you will find these research summaries helpful and, for the benefit of those of you who were unable to attend, they afford an excellent insight into the overall value of this research program.

Sincerely,

J. T. Seawell
Project Manager
Vinyl Chloride Research

JTS:ep
Enclosure

THE WRITER'S DIRECT DIAL NUMBER IS (202) 328-4258

UNIVERSITY OF LOUISVILLE

CHEMICAL CARCINOGENESIS MULTI-DISCIPLINARY RESEARCH GROUP

I. OVERVIEW

Slide 1.

The University of Louisville has a multi-disciplinary approach to the study of chemical carcinogenesis, especially, chemical monomers. Vinyl Chloride was one of the first chemical monomers under study; we combined a cancer control program which designed and implemented a prospective medical surveillance system for identifying occupationally related chemical cancer (as shown on the left of the slide) with a clinical research group which worked closely with the medical surveillance program in developing better techniques for detection and prevention. Basic research in the molecular structure and pathogenesis of carcinogens, especially chemical monomers (as shown on the right) made the third link in our multi-disciplinary approach to the study of chemical carcinogenesis.

Slide 1

MULTIDISCIPLINARY APPROACH TO CHEMICAL CARCINOGENESIS.

CANCER CONTROL PROGRAM	APPLIED CLINICAL INVESTIGATION	BASIC RESEARCH	
INYL CHLORIDE NGIOSARCOMA	MEDICAL SURVEILLANCE SYSTEMS	DETECTION AND PREVENTION SYSTEMS	PATHOGENESIS OF CARCINOGENS	MOLECULAR STRUCTURE VINYL CHLORI

SLIDE 2.

This simply outlines in more detail the various components of the cancer control program which include a screening program, a data bank, an epidemiological study system, educational programs, counseling and rehabilitation programs, and the treatment program.

Slide 2

VINYL CHLORIDE
ANGIOSARCOMA

CANCER CONTROL PROGRAM

MEDICAL SURVEILLANCE SYSTEMS

1. SCREENING PROGRAM
2. DATA BANK
3. EDUCATIONAL PROGRAMS
4. COUNSELING & REHABILITATION
5. TREATMENT PROGRAM

APPLIED CLINICAL INVESTIGATION

DETECTION AND PREVENTION SYSTEMS

BASIC RESEARCH

PATHOGENESIS OF THE CARCINOGEN (S)

MOLECULAR STRUCTURE
VINYL CHLORIDE

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SLIDE 3.

This illustrates the past clinical investigations done in the detection and prevention systems. They cover a variety of areas such as #1, looking at the appearance of normal human bile acids as a means of determining liver function to the identification of potential chemical carcinogens and their metabolites by new bacterial systems covering the gamut from clinical to basic research in the area of detection and prevention.

Slide 3

VINYL CHLORIDE

ANGIOSARCOMA

CANCER CONTROL PROGRAM

MEDICAL SURVEILLANCE SYSTEMS

APPLIED CLINICAL INVESTIGATION

DETECTION AND PREVENTION SYSTEMS

1. BILE ACID CLEARANCE
2. BLOOD/TISSUE ENZYMES
3. LEUKOCYTE ADHERENCE INHIBITION
4. HISTOCOMPATIBLE (HL-A) IDENTIFICATION
5. URINARY GLYCOSAMINOGLYCANS
6. TISSUE ANTIGENIC SYSTEMS
7. HISTOLOGICAL/ELECTRON MICROSCOPIC CHARACTERIZATION
8. MULTIVARIANT ANALYSIS FOR END PRODUCTS
9. IDENTIFICATION OF POTENTIAL CHEMICAL CARCINOGENS

BASIC RESEARCH

PATHOGENESIS OF CARCINOGENS

MOLECULAR STRUCTURE

VINYL CHLORIDE

CMA 002342

SLIDE 4.

The fourth slide illustrates some basic science research being conducted in the pathogenesis of carcinogens. In this case, they are numbered from 9 through 1 indicating those areas of research most related to the clinical area such as looking for tissue antigen and antibody detection as a means of screening to the most basic one; that is looking for industrial vinyl monomers by mass spectrometry studies and their metabolites in the carcinogenic process. These studies are presented in more detail further on. These slides simply illustrate that the inter-disciplinary group is investigating chemical monomers from molecular structure aspects to clinical application.

VINYL CHLORIDE ANGIOSARCOMA

CANCER CONTROL PROGRAM

MEDICAL SURVEILLANCE SYSTEMS

APPLIED CLINICAL INVESTIGATION

DETECTION AND PREVENTION SYSTEMS

BASIC RESEARCH

PATHOGENESIS OF CARCINOGENS

9. VINYL CHLORIDE TISSUE ANTIGEN/ ANTIBODY DETECTION
8. HEPATIC FIBROSIS IN CHEMICAL CARCINOGENESIS
7. VINYL CHLORIDE CARCINOGENESIS INDUCTION
6. GLYCOSAMINOGLYCAN IN HEPATIC ANGIOSARCOMA
5. VINYL CHLORIDE NEOPLASTIC TRANSFORMATION
4. VINYL CHLORIDE INDUCED METABOLIC ALTERATION
3. CARCINOGENESIS AND SULFUR AMINO ACID METABOLISM
2. MUTAGENESIS OF VINYL CHLORIDE AND METABOLITIES
1. INDUSTRIAL VINYL MONOMER CARCINOGENESIS

MOLECULAR STRUCTURE
VINYL CHLORIDE

ANALYSIS AND SYNTHESIS OF CHEMICALS AND THEIR USE IN BILE ASSAYS FOR THE IDENTIFICATION OF CARCINOGENIC POTENTIAL

PART A. CHEMICAL SYSTEMS OF DETECTION OF VINYL MONOMER TOXICITY

JOHN L. WONG, Ph.D.

SLIDE 1

Our study is concerned with the chemical systems of detection of the toxicity of vinyl monomers in general and vinyl chloride (VC) in particular. Most vinyl monomers are not mutagenic or carcinogenic per se, but become so after they are metabolically oxidized. They are converted to the epoxides which are highly reactive and can undergo spontaneous rearrangement to the carbonyl compounds. These primary metabolites, i.e. the epoxides and carbonyls, are electrophilic compounds and have a propensity to react with cellular nucleophiles nonenzymatically. Such reactions can be categorized into two groups, those reacting in the cytoplasm and those in the nucleus. The trapping of the epoxide and carbonyl compounds in the cytoplasmic environment by sulfhydryl compounds are generally the detoxification reactions. Whereas the primary metabolites reacting with the nucleic acid materials in the nucleus would constitute the putative action and is most likely involved in vinyl carcinogenesis. Basing on this concept, we have made advances in three areas of study: (1) The detoxification of the primary metabolites of VC in a cytoplasmic environment. Here, we have identified key intermediary metabolites and are going to identify all reaction products so that the metabolic fate of the epoxide and carbonyl can be determined as the host factors vary. (2) The putative action of the primary metabolites of VC which involves alteration of the nucleic acid constituents. We have isolated some unusual modified bases and our intention is to relate quantitatively these modifications of nucleic acids to bioassay findings. (3) The mutagenic and carcinogenic potential of industrial vinyl monomers other than vinyl chloride. We have applied controlled chemical oxidation mimicking the metabolic conditions to 14 vinyl monomers which are chosen on the basis of their industrial usage. So far 12 epoxides have been generated for testing. We have seen that this chemical study is beginning to pay off in promoting team work in unraveling the mechanism of how a vinyl monomer is being handled in a cell, which is a critical issue in industrial safety consideration.

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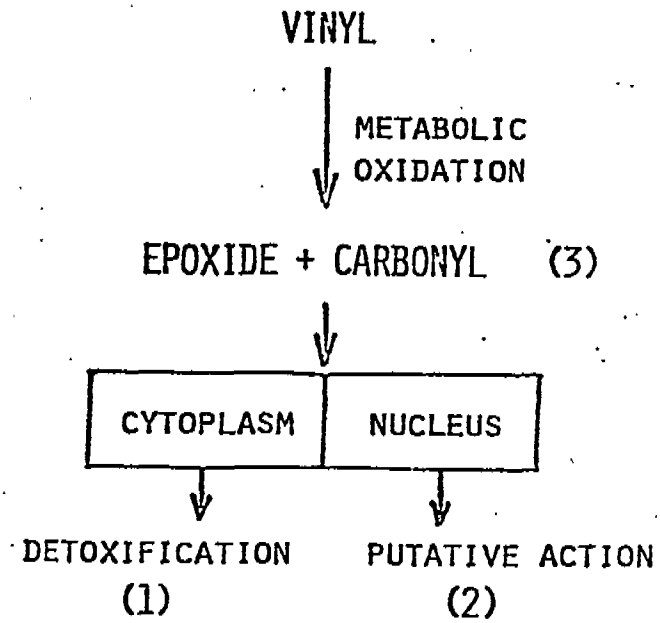
Slide 1

CHEMICAL SYSTEMS OF DETECTION OF VINYL TOXICITY



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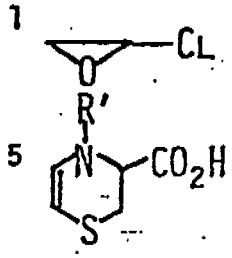
SLIDE 2

This slide shows the significant results which we have obtained pertaining to the detection of vinyl chloride detoxification products. The chlorooxirane, COR, compound 1, and its rearrangement product chloroacetaldehyde, CAA, compound 2, are the two most commonly hypothesized primary metabolites of vinyl chloride. The central question is how are they detoxified. Do they yield the same products or different ones? We have studied their reaction with sulfhydryl compounds 3,4-dichlorobenzenethiol and N-acetylcysteine. The benzenethiol was used by the Stockholm group in a priliminary study to detect the formation of CAA and COR from VC. The cysteine derivative is a cellular sulfhydryl component as well as a close analog of glutathione. In the case of COR and benzenethiol, the sulfur-conjugation product S-acetaldehyde, compound 3, is formed. However, CAA and benzenethiol forms the hemithioacetal compound 4. These two reactions are distinctly different. The formation of hemithioacetal is reversible but that of the S-acetaldehyde is not. With N-acetylcyteine, both COR and CAA yield the same cyclic condensation product, a dihydrothiazenecarboxylic acid, compound 5. This thiazene is a multi-step reaction product. Its isolation may have solved the mystery of an unknown urinary metabolite as well as the origin of the identified urinary metabolites: S-acetic acid, Compound 6, and S-ethylalcohol, Compound 7. In the Dow paper reporting the fate of ^{14}C labeled vinyl chloride in rats, there is also a major metabolite accounting for ~35% of the ^{14}C label in urine which has not been identified. We believe that this thiazene compound is a key intermediary metabolite, that it can undergo further metabolic processing to yield compounds 6 and 7, and may itself be present in the urine. These results as presented here will enable us to undertake a more comprehensive detection study of all the vinyl chloride detoxification products in biological specimens.

DETOXIFICATION OF VINYL CHLORIDE

PRIMARY METABOLITES	COR[1] ⇓	CAA[2] ⇓
INTERMEDIARY METABOLITES	S—ACETALDEHYDE[3] THIAZENES[5]	HEMITHIOACETAL[4] THIAZENES[5]
URINARY METABOLITES	S—ACETIC ACID[6]	S—ETHYL ALCOHOL[7] CHLOROACETIC ACID[8]



2 $H-\overset{O}{\overset{\|}{C}}-CH_2Cl$

3 $RS-CH_2CHO$

4 $RS-\underset{OH}{CH}-CH_2Cl$

(RS FROM 3,4—DICHLOROBENZENETHIOL, N-ACETYLCYSTEINE)

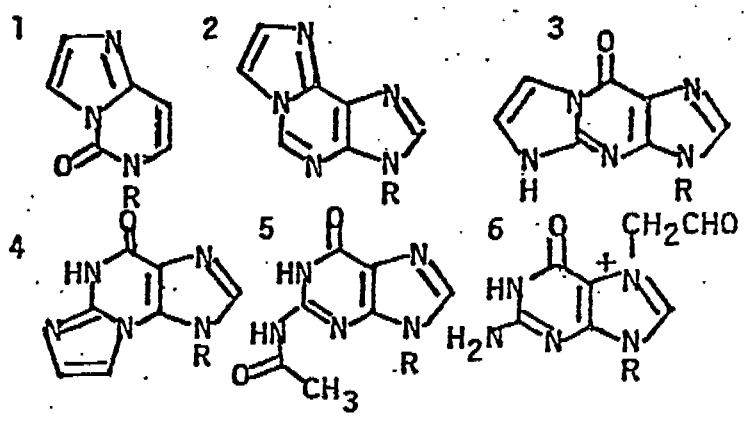
SLIDE 3

The detection study of the putative action of vinyl chloride has been started on the hypothesis that it is due to the modification of the nucleic acid materials by the primary metabolites COR and CAA. Although CAA is long known to react with nucleic acid bases such as cytosine and adenine to form the etheno derivatives, compound 1 and 2, very little is known about the reaction of CAA on the most reactive base guanine. By using a battery of modern analytical tools such as HPLC, GC-MS and FT-NMR, we have found that the guanine base in various forms, as the nucleoside, nucleotide and polyguanylic acid, gives rise to two tricyclic ethenoguanines, the linear etheno compound 3 and the angular etheno compound 4, and a third product which is possibly an acetylguanine, compound 5. These products can disrupt nucleic acid structures hence their functions by means of steric and electronic perturbation. It is also worthy of note that their fluorescence properties would allow their direct detection in a cell nucleus. The reaction of COR with the guanine base is more tricky due to the instability of COR in aqueous medium. A multitude of products are formed which we have found to be different from those of the CAA reaction. Using HPLC and NMR techniques, we have tentatively identified one major product as compound 6. It is important to note that this is the first indication that COR and CAA show different molecular events in their putative action. The significance of this information in terms of vinyl carcinogenesis will become clearer when we can correlate quantitatively the alterations in nucleic acids brought about by COR and CAA and the bioassay results of these primary metabolites.

PUTATIVE ACTION OF VINYL CHLORIDE. REACTION WITH NUCLEIC ACID BASES

CAA ETHENO—C[1], ETHENO—A[2]
L—ETHENO—G[3], A—ETHENO—G[4], ACETYL—G[5]

COR G—7—ACETALDEHYDE[6] + ...



SLIDE 4

It is now known that vinyl chloride-mediated mutagenesis in the Salmonella test requires microsomal activation. In order to extend the vinyl chloride model to scrutinize the mutagenic and carcinogenic potential of other industrial vinyl monomers, we have started a methodical screening system. The conventional Salmonella screening involves treating the vinyl monomers with the S-9 preparation which contains the mixed function oxidase system and a whole variety of other soluble enzymes prior to bacteria screening. This is sort of a black box approach, the outcome of which often is hinged on the history of the S-9 preparation. We have used mild chemical conditions, a controlled MCPBA oxidation to oxidize 14 vinyl compounds to the corresponding epoxides. They are chosen mainly on the basis of their industrial significance. These epoxides have been identified and quantitated by means of nuclear magnetic resonance technique so that the bioassay results can be related to the amounts of epoxides formed. These epoxides upon heating will rearrange to the carbonyl derivatives which can be similarly quantitated and tested. We are now vigorously pursuing this line of vinyl screening study in collaboration with Dr. Streips of the Microbiology Department. You'll be hearing from him shortly about the interesting assays which he has carried out on both the vinyl compounds and their epoxide derivatives.

To summarize, our strategy has been to fully elucidate the molecular events in the metabolism of vinyl chloride, that is, the detoxification and putative action, and use it as a model to screen the mutagenic and carcinogenic potential of other industrial vinyl monomers. This work is part of a group effort. Some of the collaboration results will now be expounded by Dr. Streips.

Slide 4

MUTAGENIC AND CARCINOGENIC POTENTIAL OF VINYL MONOMERS

$$\text{VINYL}^1 \xrightarrow[25^\circ]{\text{MCPBA}} \text{EPOXIDE} \xrightarrow{\Delta} \text{CARBONYL}$$

(1) nmr quant (1) nmr quant
(2) biol screen (2) biol screen

[1] STYRENE, BUTADIENE, ACRYLONITRILE, CROTONONITRILE, ACRYLATE, ETHYL VINYL ETHER, VINYL ACETATE, VINYL BROMIDE, VINYL FLUORIDE, VINYLIDENE CHLORIDE, VINYLIDENE BROMIDE, TRICHLOROETHYLENE, DICHLORO-DIFLUOROETHYLENE, 1,1-DICHLOROPROPENE

PART B. REACTIVITY OF CHEMICALS AND THEIR METABOLITES IN VARIOUS BACTERIAL ASSAYS - DR. ULDIS N. STREIPS, Ph.D.

With thousands of chemicals being routinely used in manufacturing processes, and new compounds being created daily for specific industrial needs, it is of great importance to assess the relative carcinogenic potential of each of these materials before they are released into the environment in quantity. When considering carcinogenesis assays, the most accurate are animal models. However, these suffer from two problems: first, they are time comsuming; second, they may not reflect the true state in human exposure. Nevertheless, to determine if a chemical is a carcinogen with our present state of knowledge requires animal testing.

There are a variety of rapid screen techniques available for chemicals. All of you are probably familiar with the Ames Salmonella reversion test. This test determines the mutagenicity of a compound. However, the correlation of mutagenesis and carcinogenesis is only accurate to about 80%. The Ames test in itself makes no prediction on the carcinogenicity of a chemical. Our laboratory also has used extensively the Bacillus repair test. This screening technique measures whether a chemical elicits enough damage to the cellular DNA to require repair for cell survival. Then by measuring the relative killing of a variety of repair-deficient strains, we can determine if the chemical is reactive to the DNA and which type of repair is required to fix this damage. A good example in Table 1 is chloroacetaldehyde (CAA), the active metabolite from vinyl chloride oxidation. This potent chemical at the concentration indicated causes no inhibition of the wild type, repair-proficient strain (w^+), or the strains lacking UV repair (hcr^-, uvr^-) and DNA polymerase I

TABLE 1

REACTIVITY OF CHEMICALS IN BACTERIAL ASSAYS

CHEMICAL	SALMONELLA REVERSION # REV/PLATE--CONTROL	SOS REPAIR % NONTREATED	SUBTILIS REPAIR ASSAY mm INHIBITION				
			W^+	REC^-	HCR^-	UVR^-	POL
CAA (0.004M)	265	15%	0	18	2	2	3
STYRENE OXIDE (0.01M)	342	100%	0	2	0	0	1
EMS (0.001M)	312	80%	1	16	1	1	1
MMS (0.001M)	355	1%	4	21	6	6	10

(pol A⁻). However, the strain missing recombination repair (rec⁻) is tremendously inhibited. This suggests that CAA interacts with DNA and that recombination repair is necessary to rectify this damage. We have done further research with this compound (Table 2) and find that very specific kinds of rec⁻ strains are inhibited by CAA. This forms the basis for much of our research in the third year on CAA. We will compare strains which are all rec⁻ but some are killed by CAA (e.g. rec A or rec B) and some are not (rec C and rec H). We will isolate enzymes and structural components from these strains which vary in their response to CAA, then relate these to known mammalian counterparts. These experiments should help to elucidate the mechanism of action of this chemical. Similar studies will be initiated for other vinyl monomers, namely styrene oxide and acrylyl nitrile.

Neither the Salmonella nor the Bacillus reversion tests directly address the carcinogenicity of the compounds tested. We have recently instituted a screen developed by Ronald Yasbin, which may approach, as closely as is possible in a bacterial system, the assay for carcinogenicity. This test will be described in detail in a forthcoming publication (U.N. Streips and R.E. Yasbin, Microbial Testers for Chemical Carcinogenesis, I.C. Felkner (ed). Marcel Dekker, N.Y.). Briefly, most investigators now envision that the carcinogenesis mechanism may be started by error-prone repair, the so-called SOS pathway. This happens when the cell cannot repair by normal means and now induces a repair system which fixes DNA lesions with no consideration for the correct template. As a result, not only has the chemical damaged the DNA of the cell, but the cell in attempting repair has created a mutational site. Therefore, any chemical which induces SOS repair is more than likely a carcinogen. The induction of SOS can be measured in several ways, but the induction of bacterial viruses is the easiest. Therefore, our test measures the extent of virus

TABLE 2

REPAIR ASSAYS WITH B. SUBTILIS*

Strains	Average inhibition in mm/8 experiments
REC A	
recA1	13.5
recA1	7.5
REC B	
recB6	13.5
recB3	9.5
recB19	16.5
recB2	16.5
recB2	11.3
recB2	16.8
REC C	
recC7	NR
REC D	
recD27	11.5
recD27	NR
REC E	
recE61	6.7
recE4	8.8
REC F	
recF7	6.1
recF18	7.4
recF15	7.5
recF15	8.42
recF16	3.1

Strains	Average inhibition in mm/8 experiments
REC H	
recH342	NR
REC	
rec-4	15.4
rec-13	5.4
MTC	
mtc-41 (caf^r)	NR
UVR	
uvr-35 (caf^r)	NR
hcr	NR
uvr	NR
WT	
wt	NR
wt	NR
wt	NR

*100mM chloroacetaldehyde used in all these experiments

induction in a cell population as a measure of SOS inducing potential. When virus is induced, the cell dies. Therefore, in Table 1 100% survival is no SOS induction, less than 100% means SOS has been induced.

At the present time several laboratories are assaying various chemicals as well as other materials by the SOS test and correlating this data to known carcinogenicity. At this time the initial preliminary evaluation is that this test may be a very accurate predictor of the carcinogenic potential of a chemical.

In Table 1 I present a comparison of the various systems. I have tested over 60 various substances in the last year by the Salmonella and Bacillus repair tests, but preliminarily only a few so far by SOS. I have chosen these four compounds for this presentation because they illustrate the variance of these microbial tests. All four compounds are mutagenic by the Salmonella test. All but styrene oxide are positive in the Bacillus repair assay, and seem to require recombination repair. Methyl methane sulfonate also is reactive toward the strain lacking polymerase I (pol A). However, the preliminary indication from the SOS repair assay demonstrates significant differences between these compounds. Both CAA and MMS are quite reactive in this assay, but styrene oxide and EMS (ethyl methane sulfonate) are essentially non-reactive. This suggests that neither styrene oxide nor EMS can induce significant levels of SOS repair. It is interesting that recent reports show EMS not to be carcinogenic while MMS is a potent carcinogen (R. Yasbin, personal communication).

It is our intention in the next funding year to finish our studies with CAA, and elucidate the several mechanisms of action of this compound. In addition we will extend our now established research techniques to thoroughly test other vinyl monomers, notably styrene oxide and acrylonitrile. We will also extend our catalogue of compounds assayed by the SOS screen and draw a more reliable conclusion on the applicability of this test for carcinogenesis screening.

THE CHARACTERIZATION OF BIOCHEMICAL ENZYMATIC CHANGES OCCURRING WITH PROLONGED VINYL CHLORIDE EXPOSURE IN ANIMAL AND MAN

J.T. Du, Ph.D., and C.H. Tamburro, M.D.

Conventional clinical biochemical studies have not been shown to be an effective early indicator of chemical injury to the liver. Little is known about the earliest cellular enzymatic changes with prolonged exposure to chemical monomers. An initial vinyl chloride exposure experiment was conducted to examine sequentially the early enzymatic changes in cellular function of the liver. Animals were exposed from 10,000 - 20,000 ppm from 14 to 137 hours, of total exposure being exposed five days a week. Conventional clinical biochemical studies and subcellular enzyme markers were studied. These included: (1) microsomal enzymes related to vinyl chloride metabolism: P450, NADPH-cytochrome C reductase, and mixed function oxidase. Mitochondrial marker cytochrome C oxidase and protein metabolism was studied by looking at in vivo incorporation of tritiated leucine. In addition, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase were studied as cytosol markers as were glutathione content and glutathione reductase in relationship to detoxification. Overall results are shown in Slide 1 for conventional and subcellular enzymes.

I. CONVENTIONAL CLINICAL BIOCHEMICAL STUDIES

1.	SGOT	NO CHANGE
2.	SGPT	NO CHANGE
3.	LDH	NO CHANGE
4.	ALK PHOS	NO CHANGE
5.	BILIRUBIN	NO CHANGE
6.	TOTAL PROTEIN	NO CHANGE
7.	ALBUMIN	NO CHANGE
8.	CHOLESTEROL	NO CHANGE
9.	TRIGLYCERIDES	NO CHANGE

II. SUBCELLULAR ENZYME AND METABOLITE STUDIES IN LIVER

1. MICROSOMAL ENZYMES RELATED TO VC METABOLISM:
   - A. P-450
   - B. NADPH-CYT C REDUCTASE — NO CHANGE
   - C. MIXED FUNCTION OXIDASE

2. MITOCHONDRIAL MARKER;
   CYT C OXIDASE — NO CHANGE

3. IN VIVO INCORPORATION OF ^{3}H-LEU — NO CHANGE

4. GLUTATHIONE CONTENT — ELEVATED

5. GLUTATHIONE REDUCTASE — INCREASED

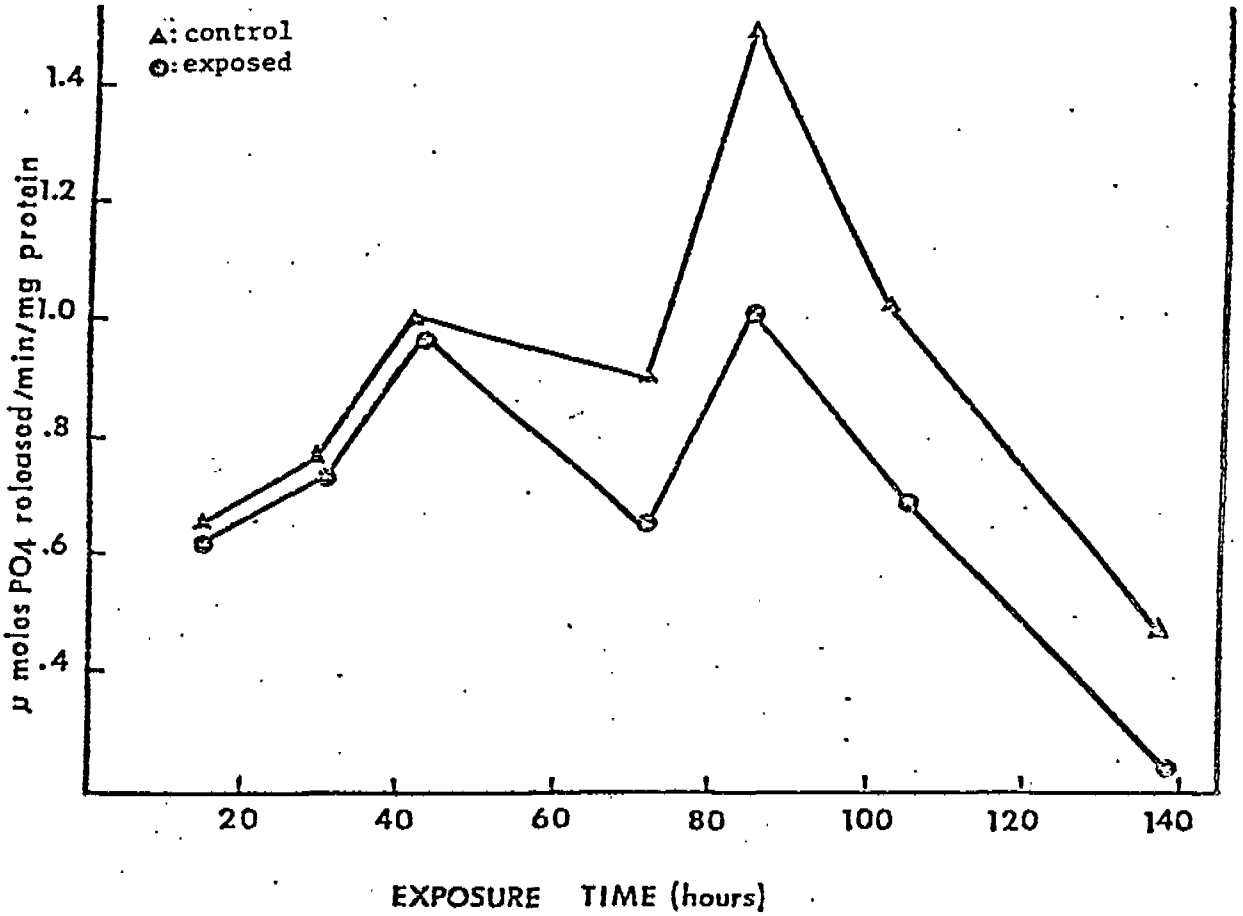
6. GLUCOSE-6-P ASE — DECREASED

7. GLUCOSE-6-P DEHYDROGENASE — INCREASED

RATS WERE EXPOSED TO 10,000-20,000 PPM VC FOR 14, 28, 42, 71, 84, 103 AND 137 HOURS.

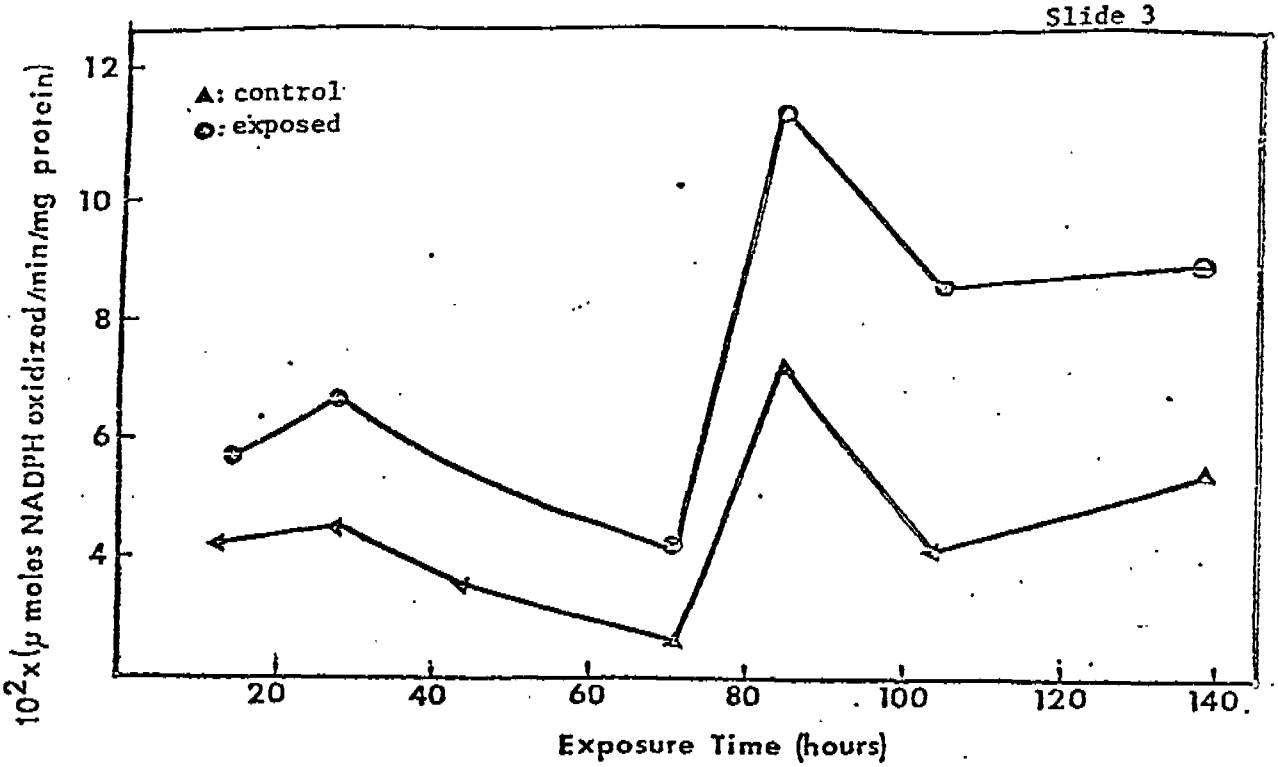
SLIDE 2, 3, 4, 5.

This illustrates an early increase in glutathione reductase activity followed closely by a fall in glucose-6-phosphatase which becomes significant after 71 hours of total exposure (Slide 3). At 100 hours, a marked increase in glucose-6-phosphate dehydrogenase (Slide 4) similar to that seen in metabolic studies by Weber and his group concerning hepatic primary cell neoplasms was found. As shown in Slide 5, Weber's molecular correlation concept of neoplasia shows an impairment of the glucose-6-phosphatase producing a decrease in gluconeogenesis and an increase in the pentose phosphate shunt pathways which eventually lead to an increase in purine biosynthesis and nucleic acid synthesis. This, presumably, leads to increased liver cell replication. These biochemical changes in the early stages of vinyl chloride exposure may well explain the histological findings in humans exposed to vinyl chloride which appear to be characteristic of vinyl chloride exposure. These histological findings of focal nodular hyperplasia of the hepatocytes may well be the histological reflection of a shift in gluconeogenesis and an increase in nucleic acid synthesis, induced by a mechanism not yet completely understood during the early phases of vinyl chloride exposure. If one were to carry this to a logical conclusion one would presume that the hepatocyte would eventually develop into a malignant cell with continuous regeneration and exposure to carcinogens. The fact that the primary hepatocyte does not become malignant in the adult rat, but does become malignant in the newborn rat with similar vinyl chloride exposure, led us to study the detoxifying capabilities of the hepatocyte versus the sinusoidal lining cells. During this time, there were no significant differences in the usual biochemical clinical studies used for screening early vinyl chloride injury.



SLIDE 2 SHOWS THE GROUP MEANS OF THE SPECIFIC ACTIVITY OF GLUCOSE-6-PHOSPHATASE WITH RESPECT TO TIME OF EXPOSURE. THE CONFIDENCE INTERVALS FOR GROUP MEAN DIFFERENCES ON THE ACTIVITIES OF ENZYMES DISCUSSED ARE BASED ON THE ERROR MEAN SQUARE FROM A TWO FACTOR ANALYSIS OF VARIANCE WITH INTERACTION. THE TWO MAIN EFFECTS BEING TIME IN HOURS AND EXPOSURE-NONEXPOSURE. THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN THE EXPOSED AND CONTROL GROUPS FOR THE FIRST THREE TIME POINTS. HOWEVER, THE MEAN LEVEL OF THE EXPOSED GROUP IS SIGNIFICANTLY LESS THAN THAT OF THE CONTROL AFTER 71 HOURS OF EXPOSURE.

Slide 3



SLIDE 3 SHOWS THE GROUP MEANS OF THE SPECIFIC ACTIVITY OF GLUTATHIONE REDUCTASE WITH RESPECT TO TIME OF EXPOSURE. THERE IS A SIGNIFICANT INCREASE THROUGHOUT THE ENTIRE EXPERIMENT.

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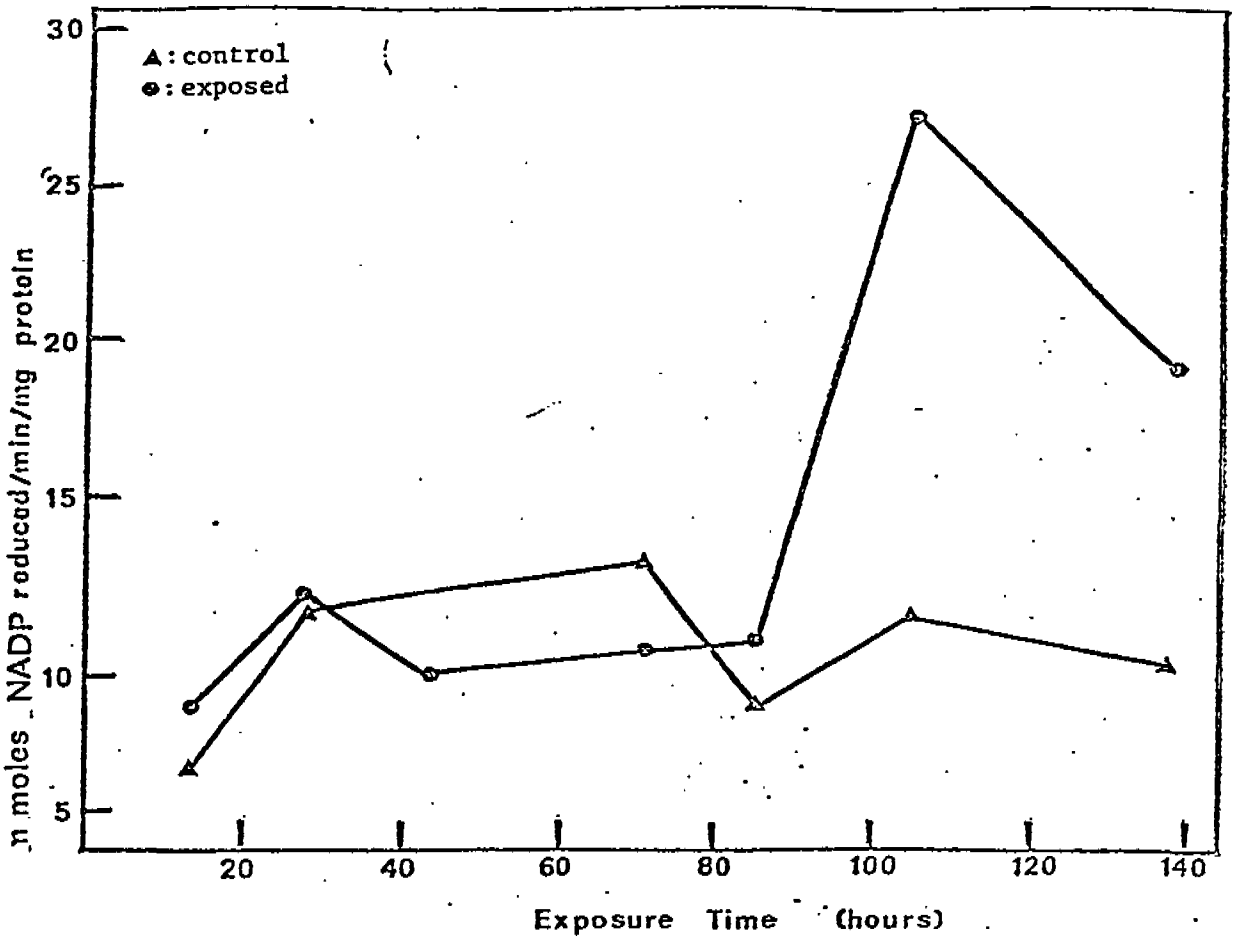
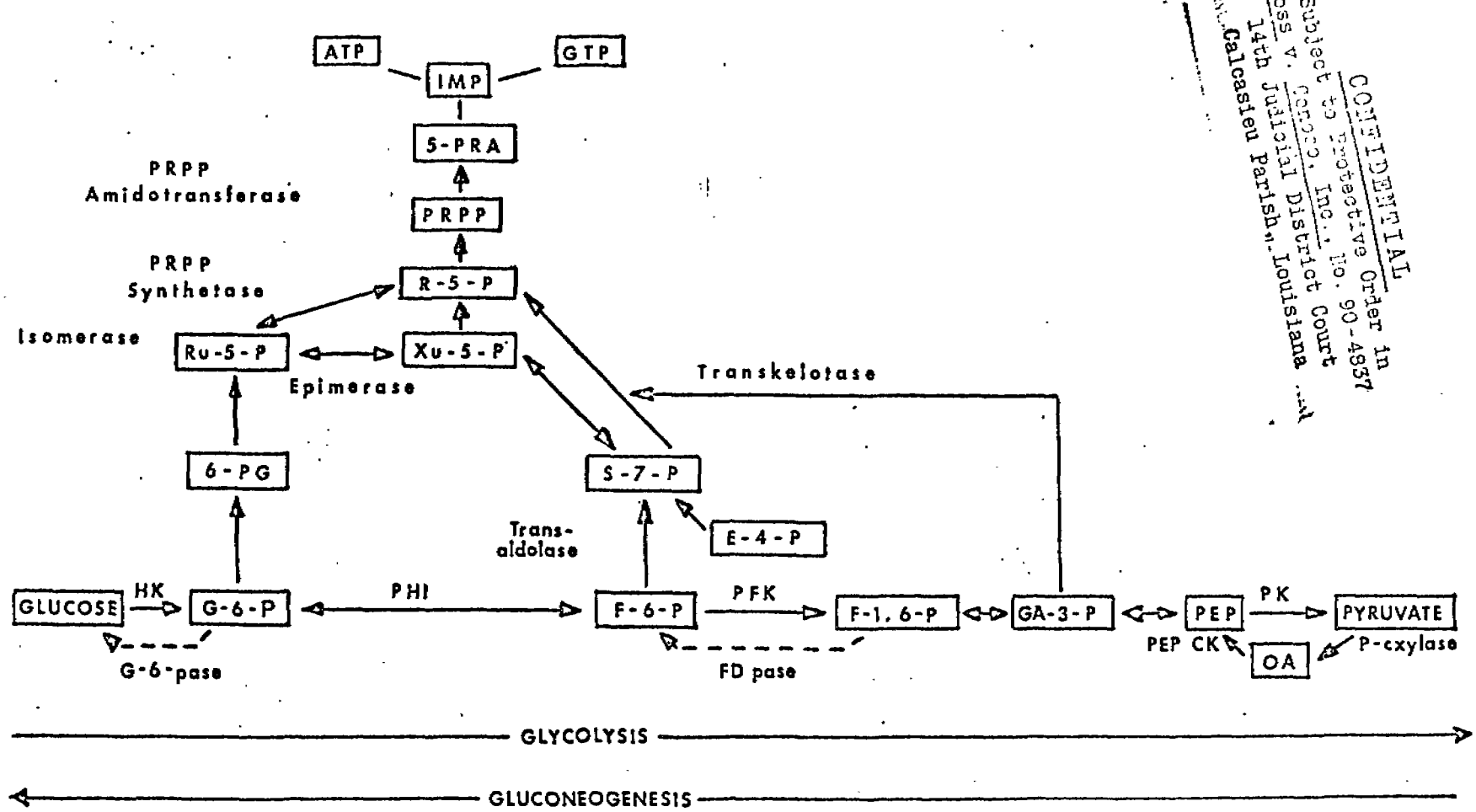


SLIDE 4 SHOWS THE GROUP MEANS OF THE SPECIFIC ACTIVITY OF GULCOSE-6-PHOSPHATE DEHYDROGENASE WITH RESPECT TO TIME OF EXPOSURE. THERE IS A SIGNIFICANT INCREASE AFTER ABOUT 100 HOURS OF EXPOSURE.

WEBER'S MOLECULAR CORRELATION CONCEPT OF NEOPLASIA



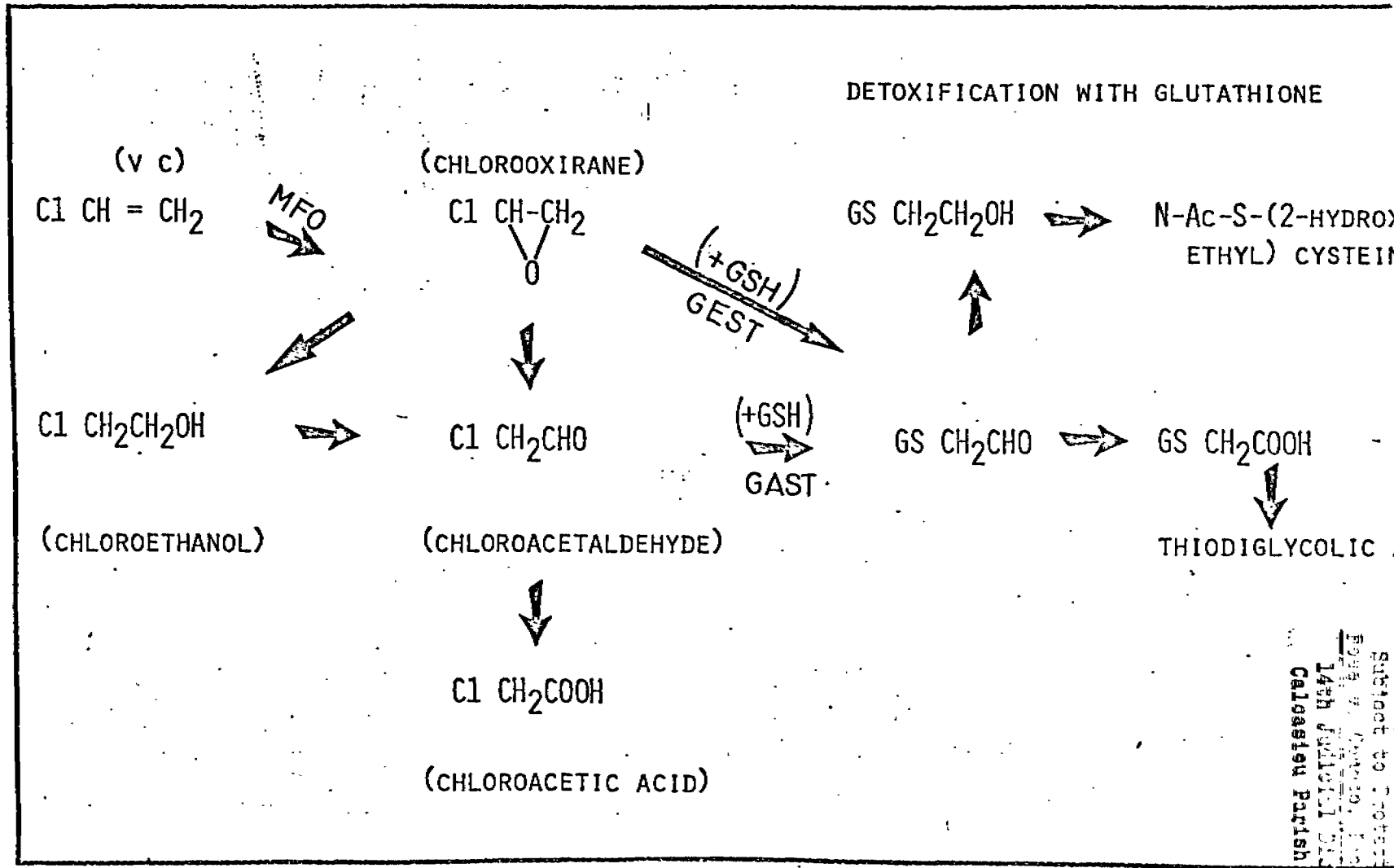
VINYL CHLORIDE OXIDATION AND DETOXIFICATION

J.T. Du, Ph.D., and C.H. Tamburro, M.D.

The second phase of study of the biochemical changes associated with vinyl choloride exposure were directed at oxidation and detoxification. Sprague-Dawley rats were randomly exposed to controls and vinyl chloride exposure at 28,000 ppm, 7 hours per day, five days per week, for a total of 210 hours of exposure, for six weeks duration. Slide 1 shows the vinyl chloride metabolic fate scheme presently under use. Vinyl chloride being metabolized at higher levels by mixed-function oxidase within the liver may be converted to chlorooxirane or the epoxide. This unstable intermediate may then spontaneously revert to form chloroethanol and the chloroethanol may further be metabolized to chloroacetaldehyde. Secondly, the chlorooxirane may be directly detoxified by GSA via the glutathione epoxide S-transferase or further oxidized to chloroacetaldehyde. Chloroacetaldehyde in turn may be shown by glutathione aralykly-S-transferase or further oxidized to chloroacetic acid. Studies by Wong and Streips have already shown that vinyl chloride, chloroethanol and chloroacetic acid have no mutagenic properties in bacteria. However, the chlorooxirane and the chloroacetaldehyde are both mutagenic. Animal studies demonstrated significant differences in the liver of vinyl chloride exposed animals who showed no physical or clinical abnormalities. These findings are listed in Slide 2. They include an elevation of non-protein sulfhydryl content, (Slide 3) glutathione reductase, (Slide 4) glutathione epoxide-S-transferase (GEST) (Slide 5) and glutathione aralykly-S-transferase (GAST) (Slide 6) concomitant with the increase in glucose-6-phosphate dehydregenase and reduction in P450 content (Slide 7).

A composite of the studies from 14 to 350 hours is shown in Slide 8 which illustrates the earliest increases occurring in glutathione reductase followed later by an increase in glutathione content. It is interesting to note that the increase in GEST occurred before the increase in GAST around 70 to 80 hours of the total exposure. This implies that the chlorooxirane accumulation is to be

handled by direct detoxification by the glutathione but with prolonged exposure more is converted to the chloroacetaldehyde which then is further detoxified by the GAST. These data are compatible with other findings of increased amounts of chloroacetic acid in the urine of individuals with very high prolonged exposures. This would imply that at initial doses the vinyl chloride is able to be metabolically handled and properly detoxified and that there may be a number of mechanisms by which the active metabolites are handled within the hepatocyte thus, preventing it from inducing DNA injury and subsequently angiosarcoma. Whether these metabolic capabilities are also present in the sinusoidal lining cells has lead us to our more recent studies.



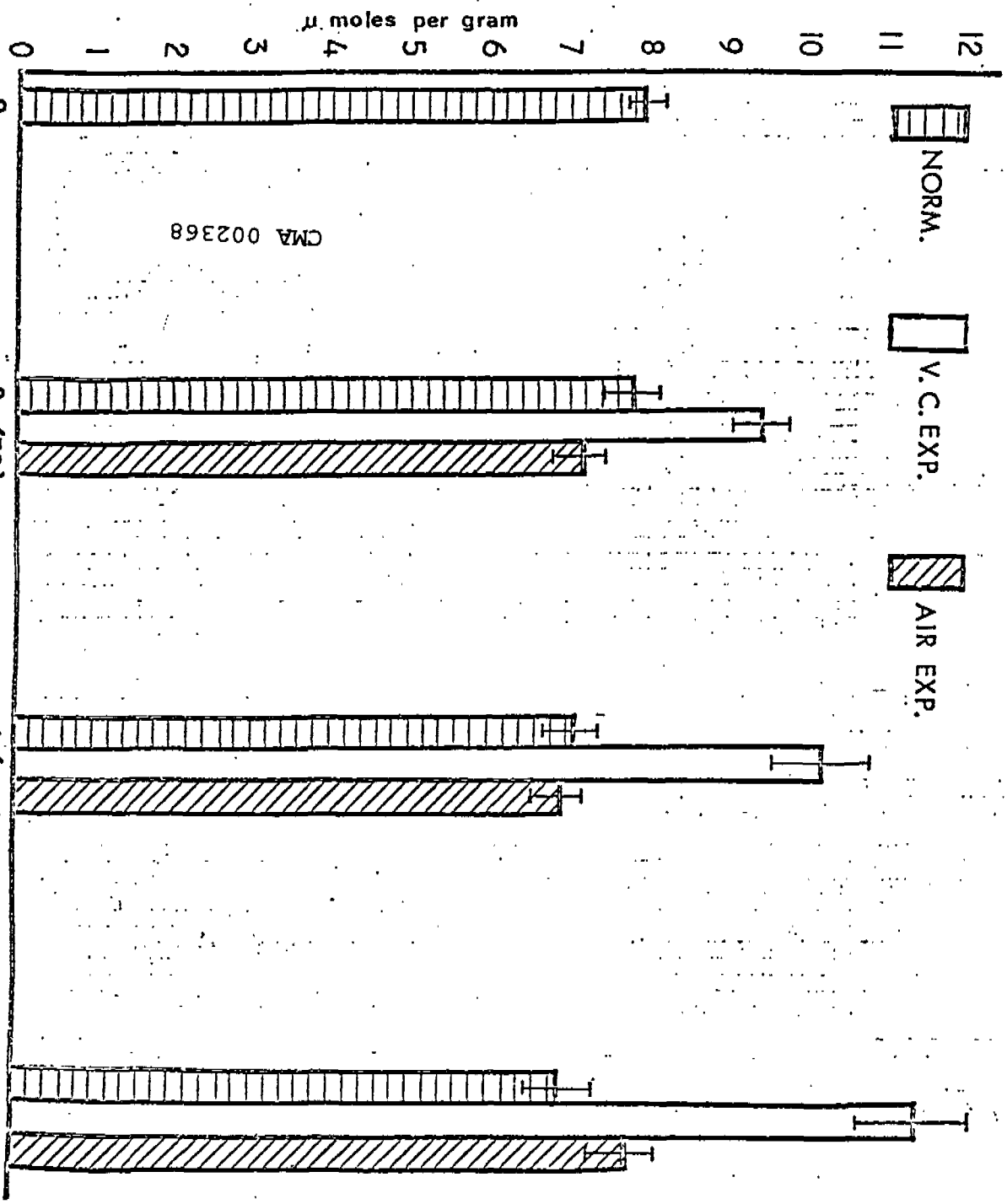
VINYL CHLORIDE METABOLIC FATE

RESULTS

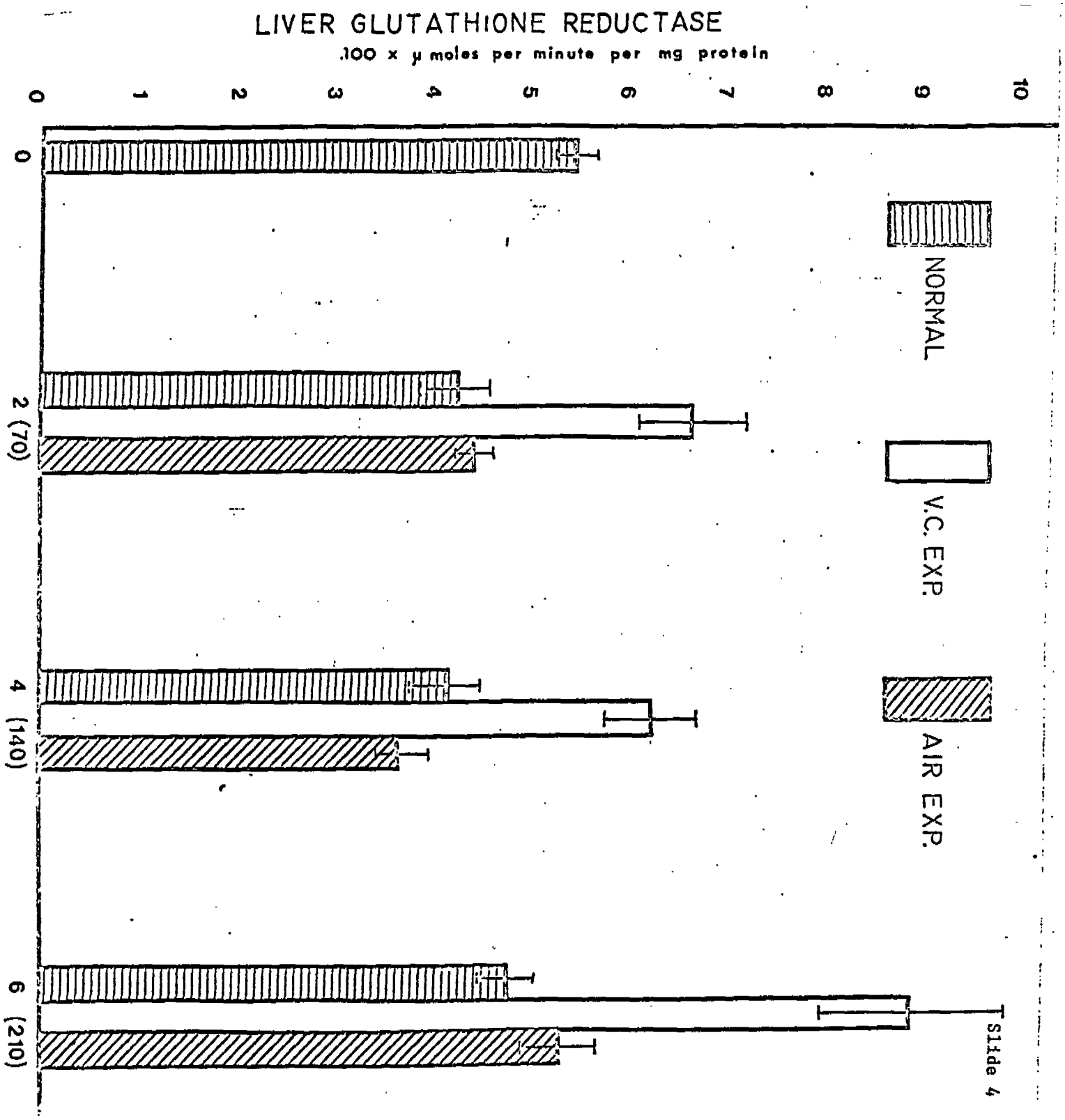
IN COMPARISON WITH THE TWO CONTROL GROUPS, THE FOLLOWING STATISTICALLY SIGNIFICANT DIFFERENCES ($P < 0.05$) WERE FOUND IN THE LIVER OF THE VINYL CHLORIDE EXPOSED BEFORE ANY SIGN OF PHYSICAL OR CLINICAL ABNORMALITIES WERE SEEN.

1. AN ELEVATION OF NON-PROTEIN SULFHYDRYL CONTENT.
2. AN ELEVATION OF GLUTATHIONE REDUCTASE.
3. AN ELEVATION OF GLUTATHIONE EPOXIDE -S- TRANSFERASE (GEST).
4. AN ELEVATION OF GLUTATHIONE ARALKYL -S- TRANSFERASE (GAST).
5. A REDUCTION OF P-450 CONTENT.
6. AN ELEVATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

NON-PROTEIN SULFHYDRYL GROUP CONTENT IN LIVER



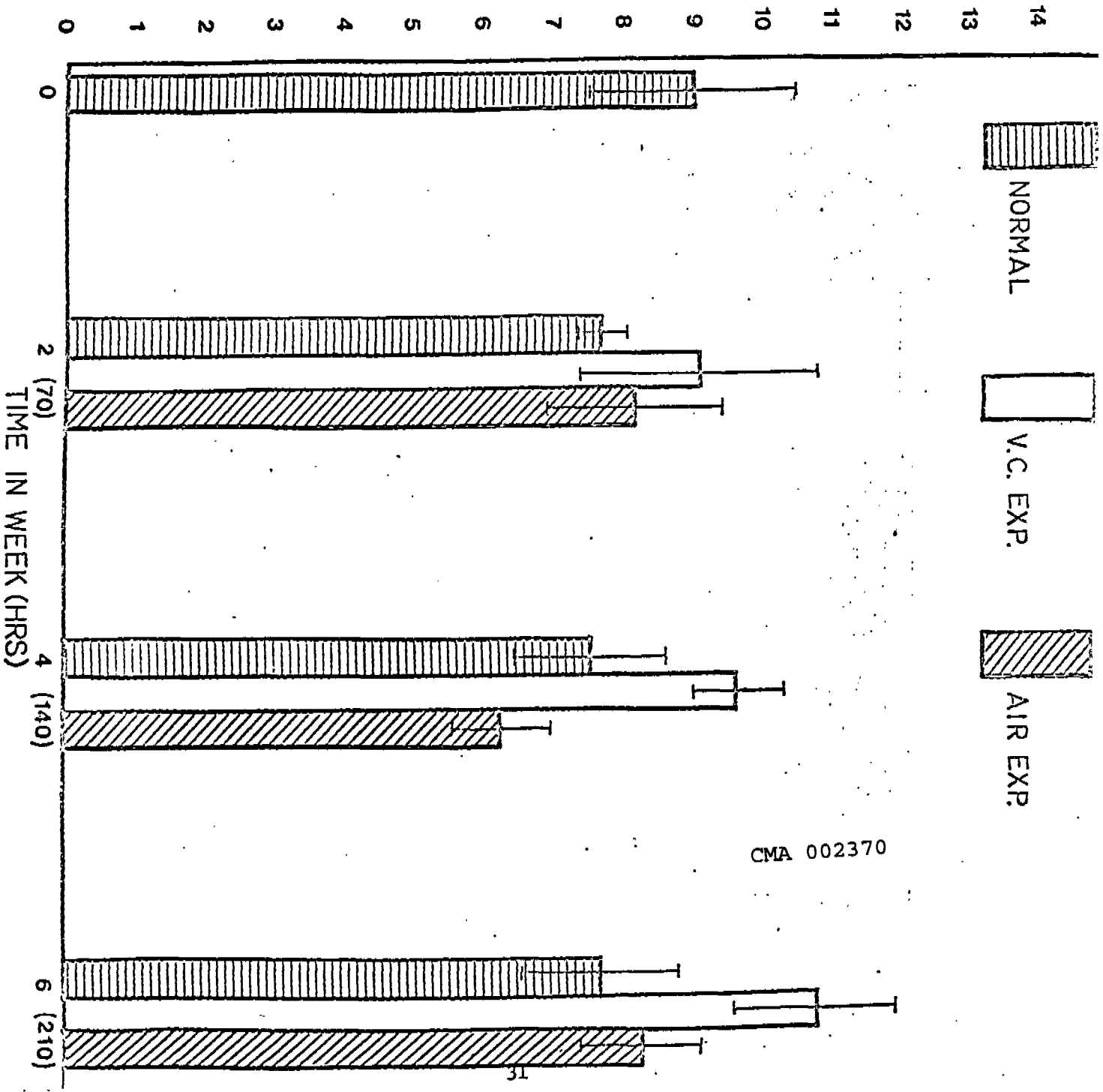
Slide 3



LIVER

GLUTATHIONE EPOXIDE -S- TRANSFERASE

100 x μmoles per minute per mg protein



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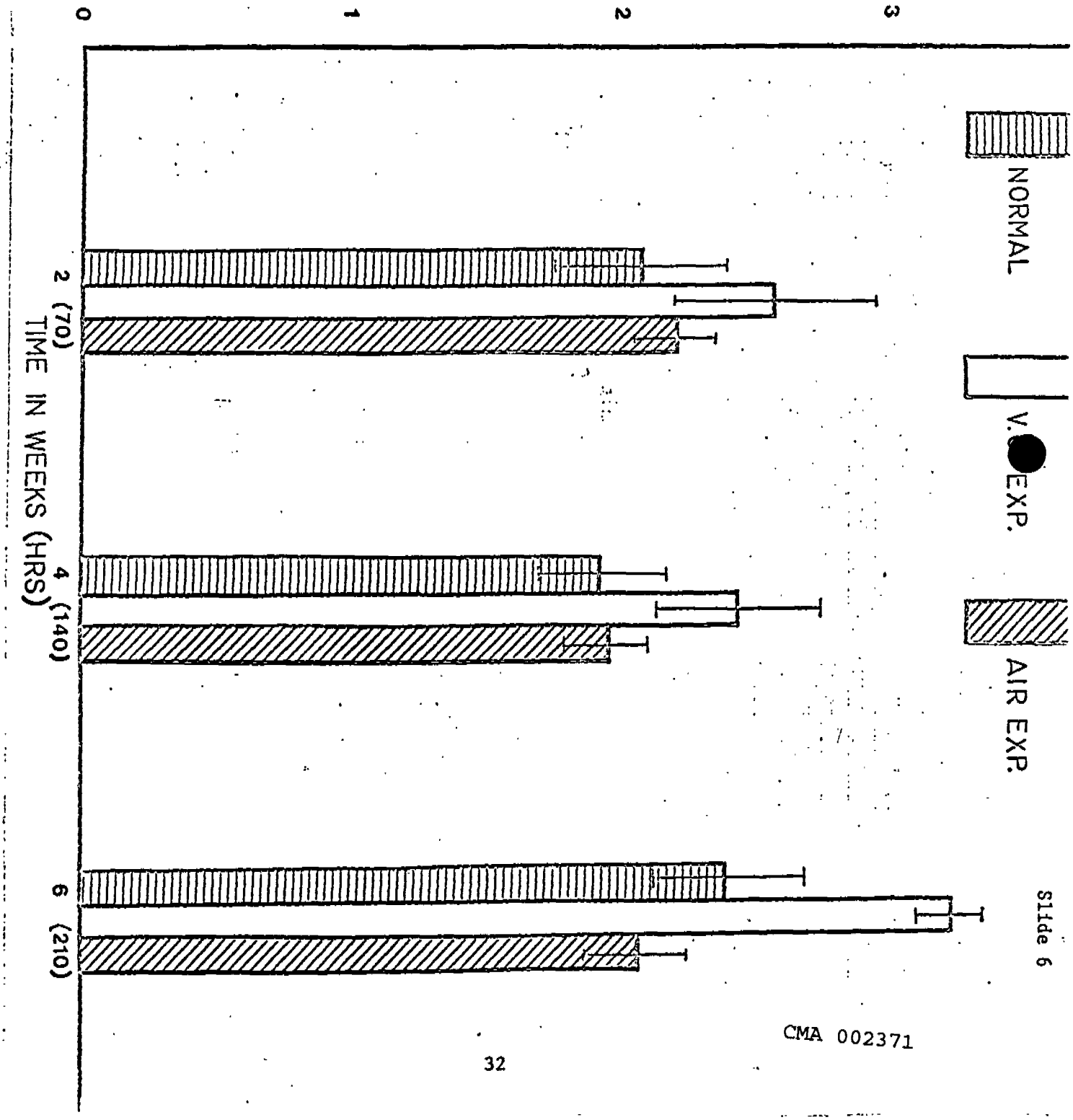
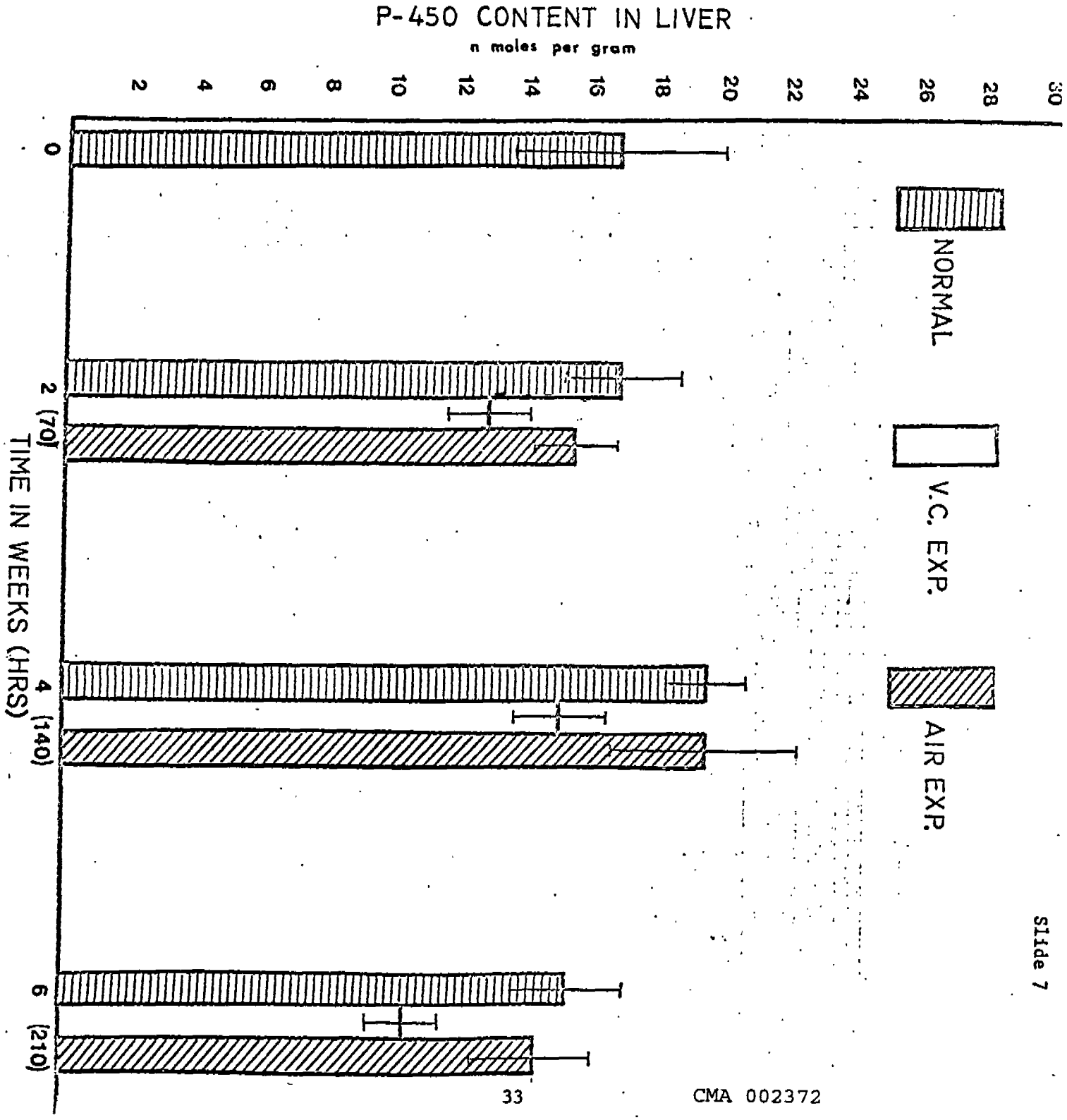
LIVER

GLUTATHIONE ARALKYL - S - TRANSFERASE

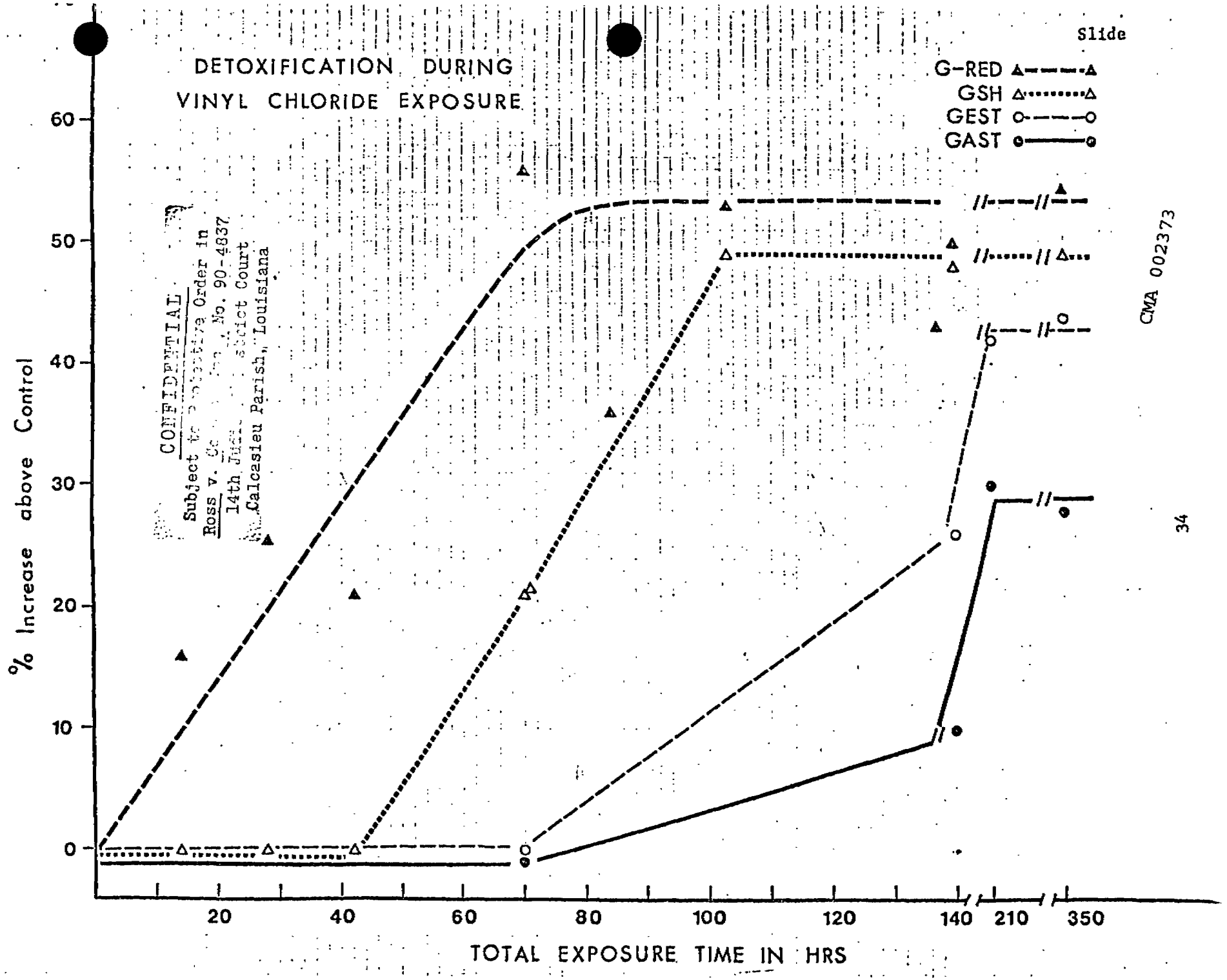
10 x μ moles per minute per mg protein



Slide 6



Slide 7


DETOXIFICATION DURING
VINYL CHLORIDE EXPOSURE
Slide
G-RED
GSH
GEST
GAST
CONFIDENTIAL
Subject to Protective Order in
Ross v. [illegible], No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana
CMA 002373
34
% Increase above Control
0
10
20
30
40
50
60
20
40
60
80
100
120
140
210
350
TOTAL EXPOSURE TIME IN HRS

IMMUNOPATHOLOGY OF VC-ASSOCIATED LIVER ANGIOSARCOMA

AND THE EFFECTS OF INDUSTRIAL CHEMICALS ON ANTIGEN ANTIBODIES

E. Espinosa, M.D.

It has long been known that chemicals can modify antibodies in antigens of serums, and tissues. The measuring of these changes, hopefully, would lead to an early detection of the chemical-associated diseases and provide an approach to study the pathogenesis of such disorders. Chemicals or their metabolites can modify antibodies or antigens due to their strong interaction with proteins affecting the quality and quantity of these proteins. Secondly, their measurement may lead to early detection and understanding of the pathogenesis of this injury. The first slide summarized briefly our immunopathologic observations in VC-associated angiosarcoma.

Slide 1

IMMUNOPATHOLOGY OF VC-ASSOCIATED LIVER ANGIOSARCOMA

A. Antigens investigated in liver angiosarcoma:
   1. Tumor-associated antigen
   2. Antigens shared with tissues: CTA-2, STA-3
   3. Antigens shared with bile: BT-1, BT-2, BT-3
   4. Antigens with liver specificity

B. Tumor bound IgG

C. Antigens deleted

Firstly, four classes of protein antigens were detected by immunodiffusion and immunofluorescent in the liver tumors: a tumor associated antigen; antigens shared with tissues; antigens shared with bile; and antigens with liver specificity. Studies of the physicochemical properties of these antigens and their presence in circulation have been completed and reported (on some of these antigens, while additional studies are being continued on the other antigens).

We have also reported the presence of IgG binding to liver tumors, indicating that these tumors do produce antigens which stimulate B cell antibodies. Work is still in progress to identify the antigen which stimulates such antibody production.

Our third major finding is related to the deletion of normal antigens in the angiosarcoma tumor. This deletion of normal antigens has recently stimulated similar analysis in chemically induced hepatomas in rats. Similarly to the angiosarcoma, two tissue antigens were found to be missing in these hepatomas. Studies are now being conducted concerning the properties of these antigens and the significance of their deletion. It may well be that the deletion of certain antigens may be a more sensitive indicator of tumor development.

Table 1 demonstrates the frequency of circulating tissue antigens in various clinical disorders in patients with various liver and non-liver diseases. One finds that circulating tissue antigen-2 (CTA-2) has been found most frequently in acute hepatocellular injury such as in acute viral hepatitis and acute alcoholic hepatitis but to a lesser degree in chronic forms of disease such as cirrhosis and carcinoma and not at all in those with liver angiosarcoma. This data would lend further support to the concept that the presence of tissue bound antigens or their deletion are better markers of tumor development than those in circulation.

TABLE 1

Frequency of CTA-2 in patients with liver diseases, other diseases and healthy subjects

Patients	No. tested	No. with CTA-2
Acute hepatitis:		
Infectious	29	23 (79%)
Serum	9	7 (77%)
Alcoholic	8	5 (62%)
Liver cirrhosis	28	8 (28%)
Obstructive jaundice	5	1 (20%)
Liver angiosarcoma	3	0 (0%)
Pancreatitis	8	2 (25%)
Pneumonia	22	2 (9%)
Congestive heart failure	39	8 (20%)
Myocardial infarction	23	3 (13%)
Carcinomas	35	8 (23%)
Newborns	12	0 (0%)
Healthy controls	48	0 (0%)

A SEARCH FOR EVIDENCE OF A VINYL CHLORIDE-INDUCED TUMOR ANTIGEN

H.P. Fortwengler, M.S., and C.H. Tamburro, M.D.

New antigens arise on tumors formed as a response to carcinogens. Their presence on methylcholanthrene-induced sarcomas was discovered by Foley in 1953. This discovery in mice was verified and extended by Prehn and Main in 1957 to conclude that there were antigens peculiar to and specific for tumor tissue. Subsequently, evidence for tumor antigens was found in humans by the Hellstroms, Vankey, Halliday and Maluish, Thompson and others. The majority of the evidence suggested that the tumor antigens found were distinctive for each histological type of tumor.

In an effort to capitalize on the well established fact that the body mounts an immune reaction to cancer, Fortwengler's group is endeavoring to find evidence of these specific immune reactions and then utilize them to develop a relatively specific test for possible VC induced tumor development. Lymphocytes (the cells responsible for immunity) from the individual tested are being isolated by density gradient centrifugation. These cells are then grown in the presence of a liver reagent prepared from either a normal individual or an individual who had angiosarcoma. A positive reaction is a three times increased incorporation of H^3-thymidine into stimulated cultures.

A comparison of the responses between vinyl chloride plant workers and normal non-vinyl chloride workers indicated that there were many people in the general population having reactivity to tissue antigens irrespective of whether it was from angio or normal liver. Non-specific reactions of this type may be due to sensitization by "natural" means, injections of human or animal substances, transfusions, etc. The reactions of lymphocytes to normal and angio liver were quantitated.

A comparison of the responses to angio or normal liver by VC workers according to known VC exposure was made. The VC workers were grouped as either

high exposure or low exposure depending on whether they had exposure above the median plant exposure. As can be seen in Figure 1, no statistical differences were noted between the two groups.

On the chance that the non-specific reactions may be obscuring the specific reactions all those VC workers having reactions to both angio liver and normal liver were grouped (see Table 1). Those with reactions to normal liver only were grouped as were those with no reactions. That left only those with reactions to angio liver. As can be seen in Table 1, the only reactions obtained with possible specific tumor reactivity were found in the group of VC workers.

These results, although provocative, must be extended by testing additional VC workers but more importantly by testing a comparison group of individuals having no known VC exposure. Our plans for the future include an expansion of the above data and a slight reorganization of experimental design to ensure maximal statistical strength from the data obtained.

Figure 1

LYMPHOCYTE RESPONSE OF VC WORKERS ACCORDING TO EXPOSURE

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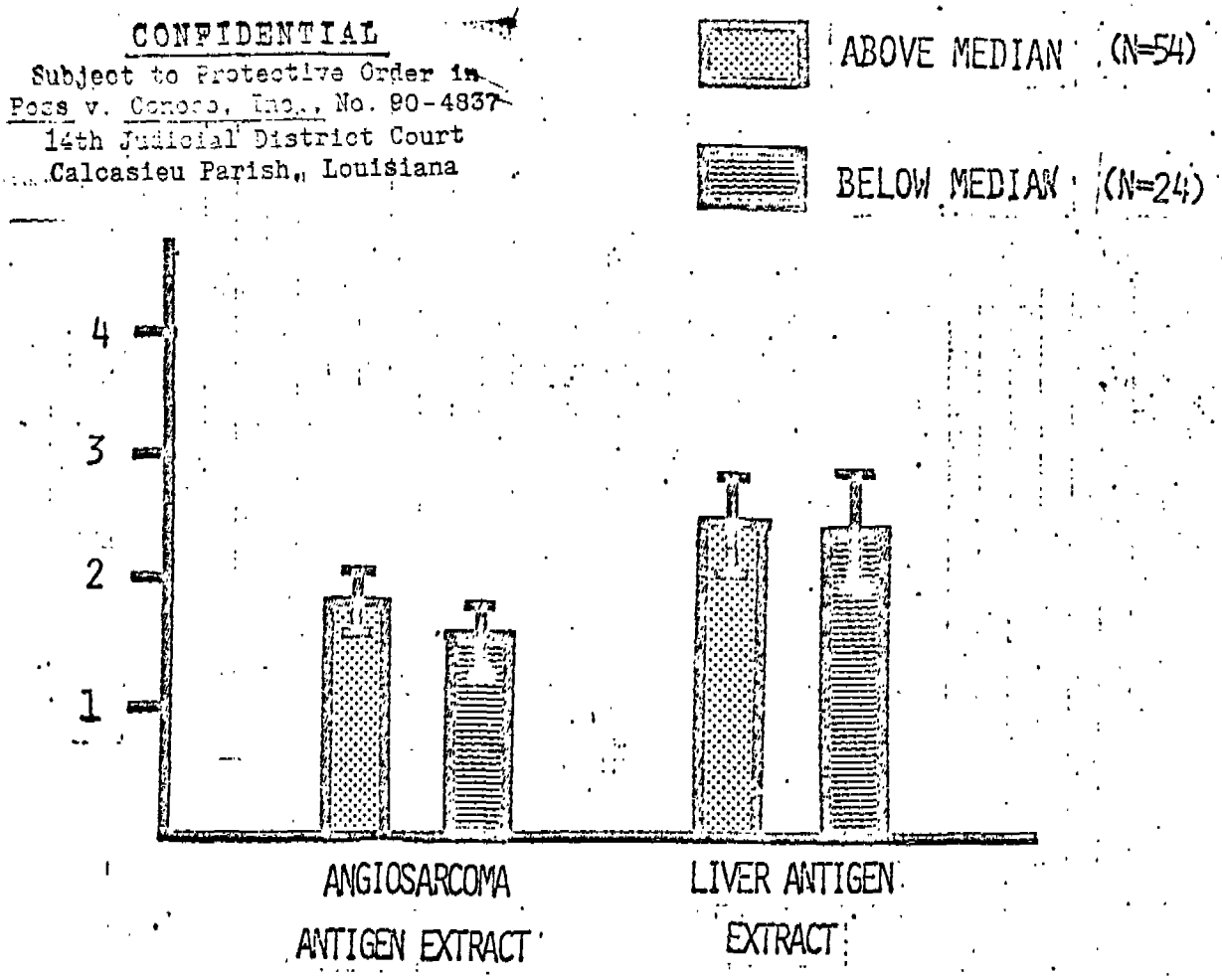

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(Table 1)

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LYMPHOCYTE REACTIVITY TO TUMOR AND NORMAL TISSUE PANEL

	VINYL CHLORIDE WORKERS		NON-VINYL CHLORIDE WORKERS		INTERPRETATION OF RESULTS
	n	percentage	n	percentage	
ANGIO LIVER	6	16	0	0	Individuals with possible specific anti-tumor reactivities.
NORMAL AND ANGIO LIVER	7	18	8	57	Individuals with nonspecific reactivities masking any possible specific reactivities.
NORMAL LIVER	5	13	1	7	Individuals with nonspecific reactivities.
NONREACTIVE	20	53	5	36	Individuals with no tissue reactivities.
TOTAL	38	100	14	100	

HLA FREQUENCIES IN VINYL CHLORIDE WORKERS

H.P. Fortwengler, M.S., and C.H. Tamburro, M.D.

Mulvihill in the Journal of the National Cancer Institute in 1976 stated that a challenge to science is to develop a means of screening potential employees for abnormal genotypes that predispose them to neoplasia after occupational exposure which are harmless to normal genotypes.

An increased incidence of certain HLA types has been shown to be associated with susceptibility to various diseases. HLA-A27 antigen has been found more often in workers suspected of having the occupational disease asbestosis than among a control population.

HLA tissue typing procedures have been initiated in our laboratory and we have typed approximately 300 individuals from the Louisville vinyl chloride polymerization plant. Individuals do not change their genetic complement of HLA antigens, so the determinations need not be repeated periodically as in various clinical assays. Tissue typing for 11 HLA-A antigens and 16 HLA-B antigens and their possible increased association with angiosarcoma or other chemically related diseases is about one-third completed.

A comparison between those employees found to have liver disease, labeled "Pallet Plant", and those without liver disease, labeled "Non-Pallet Plant", may be seen in Tables 1 and 2.* Although the number of individuals tested is not as yet large enough to allow statistical significance to emerge, potential difference in frequencies may be seen at A9, B15, B27, and B17.

As an additional procedural and population check, our compiled data is being compared with the frequencies obtained by two other large HLA studies. Frequencies of the healthy individuals studied by Scott et al, 1977, and the World Health Organization compare favorably to the frequencies found in the University of Louisville study.

*These are individuals who because of their liver disease, were initially reassigned to a shop manufacturing wooden pallets.

It has been clearly established that the larger the population studied the greater the chances of obtaining statistically significant data. The accumulation of HLA data on the majority of the VC population plant workers will take an additional two years as originally anticipated. This is necessary so that the strongest statistical analysis can be performed upon the completion of all HLA testing.

HLA - A FREQUENCIES IN VINYL CHLORIDE WORKERS

HLA - A ANTIGENS*	HEALTHY CONTROLS**	WHO 1975 WORKSHOP	UL/PALLET PLANT***	UL/NON-PALLET PLANT
A1	34	32	38	30
A2	51	49	58	53
A3	23	22	20	18
A9	16	17	8	23
A10	7	13	12	7
A11	13	8	8	10
A28	7	11	20	13
A29	9	7	0	7
AW19	ND****	28	ND	ND
AW30	ND	5	8	3
AW31	ND	7	4	7
AW32	ND	8	8	7
BLANK	ND	ND	16	21
N	900	6877	24	243
TOTAL FREQ.	160%	207%	200%	199%

* CURRENTLY TYPING FOR 11 ANTIGENS
** SCOTT ET AL., 1977
*** DATA COMPILED JUNE, 1978
**** NOT DONE

HLA - B FREQUENCIES IN VINYL CHLORIDE WORKERS

HLA - B ANTIGENS*	HEALTHY CONTROLS**	1975 WHO WORKSHOP	UL/PALLET PLANT***	UL/NON-PALLET PLANT
B5	10	11	8	7
B7	31	23	22	28
B8	27	20	16	22
B12	30	24	26	25
B13	3	6	0	6
B14	5	11	12	12
B15	10	7	38	12
B18	8	9	0	2
B27	7	8	0	12
B37	ND****	5	4	1
B40	11	12	16	17
BW16	ND	12	0	2
BW21	ND	4	4	4
BW22	2	5	4	6
BW35	5	17	8	13
B17	6	7	22	10
BLANK	ND	ND	12	14
N	900	6877	24	243
TOTAL FREQ.	155%	181%	192%	193%

* CURRENTLY TYPING FOR 16 ANTIGENS
** SCOTT ET AL., 1977
*** DATA COMPILED JUNE, 1978
**** NOT DONE

EVALUATION OF IMMUNOCOMPETENCE OF HUMANS CHRONICALLY EXPOSED TO VINYL CHLORIDE

H.P. Fortwengler, M.S., and C.H. Tamburro, M.D.

It has been demonstrated that lymphoid cells (T cells) can be cytotoxic to human tumor cells and are often found decreased in cancer patients. Viola, et al, reported the development of neoplasms in rats exposed to vinyl chloride (VC). Creech and others reported the increased incidence of human angiosarcoma in VC production workers. It is the purpose of this study to determine the immunocompetence of individuals that have undergone prolonged exposure to VC monomer and have developed liver lesions which, in some instances, are thought to presage the development of angiosarcoma.

The scientific literature is replete with demonstrations of immunodepression as shown by one or more immune parameters in cancer patients at various stages of disease. There is very little information in the scientific literature concerning immunoevaluation prior to diagnosis of frank malignance. We have used immunological assays (see Table 1) that have demonstrated usefulness in indicating immunodepression in cancer patients.

These tests have been used to evaluate the immunocompetence of individuals with possible pre-malignant lesions or other liver disease as demonstrated by biopsy. A comparison of these diseased individuals with normal plant workers demonstrated nothing to suggest that the groups were immunologically different. A comparison of those individuals having high VC exposure (defined as those with lifetime exposure above the median) with individuals having low exposure (below the plant median VC exposure) demonstrated a slight pattern of immunodepression (see Fig. 1) when lymphocytes were stimulated by PHA and Con A. Preliminary evaluation of this data indicated there is no statistical significance between the high and low exposure groups when examined with student's t test. Even after additional immunological parameters were examined (see Table 2), no statistical differences could be found between the two groups.

At this time, our preliminary interpretation of these results, which will soon undergo a more complete data analysis, is that there is no residual immunological depression as a result of chronic exposure to increased levels of VC as determined by our standard battery of immunological tests.

Further computer analysis of our data in addition to concomitant correlations with data being received from clinical testing (e.g. immunoglobulin quantitation) will give us more definitive statistical results.

TABLE 1

BATTERY OF IMMUNOCOMPETENCE TESTS IN USE

TEST	REASON FOR USE
Absolute Lymphocyte Count	Gives a gross examination of the immune system
SRBC Rosettes, 4°C	Quantitates the total number and percent of T cells. (T cells can reject tumors and transplants.)
SRBC Rosettes, 33°C	Quantitates the total number and percent of an active subset of T cells
PHA Stimulation	Tests the ability of T cells to respond to a non-specific stimulant
Con A Stimulation	Tests the non-specific function of T cells; overlaps, but not identical to PHA
PWM Stimulation	Tests the non-specific function of T cells; has B cell participation
SPL Stimulation	Tests the non-specific function of T cells; has B and Null cell participation
Recall Antigenic Stimulation	
SLO PPD Candida Varidase	Determines the ability of sensitized lymphocytes to be activated by rechallenge; everyone should react with at least one of these common antigens

Figure 1

LYMPHOCYTE RESPONSE OF VC WORKERS ACCORDING TO EXPOSURE

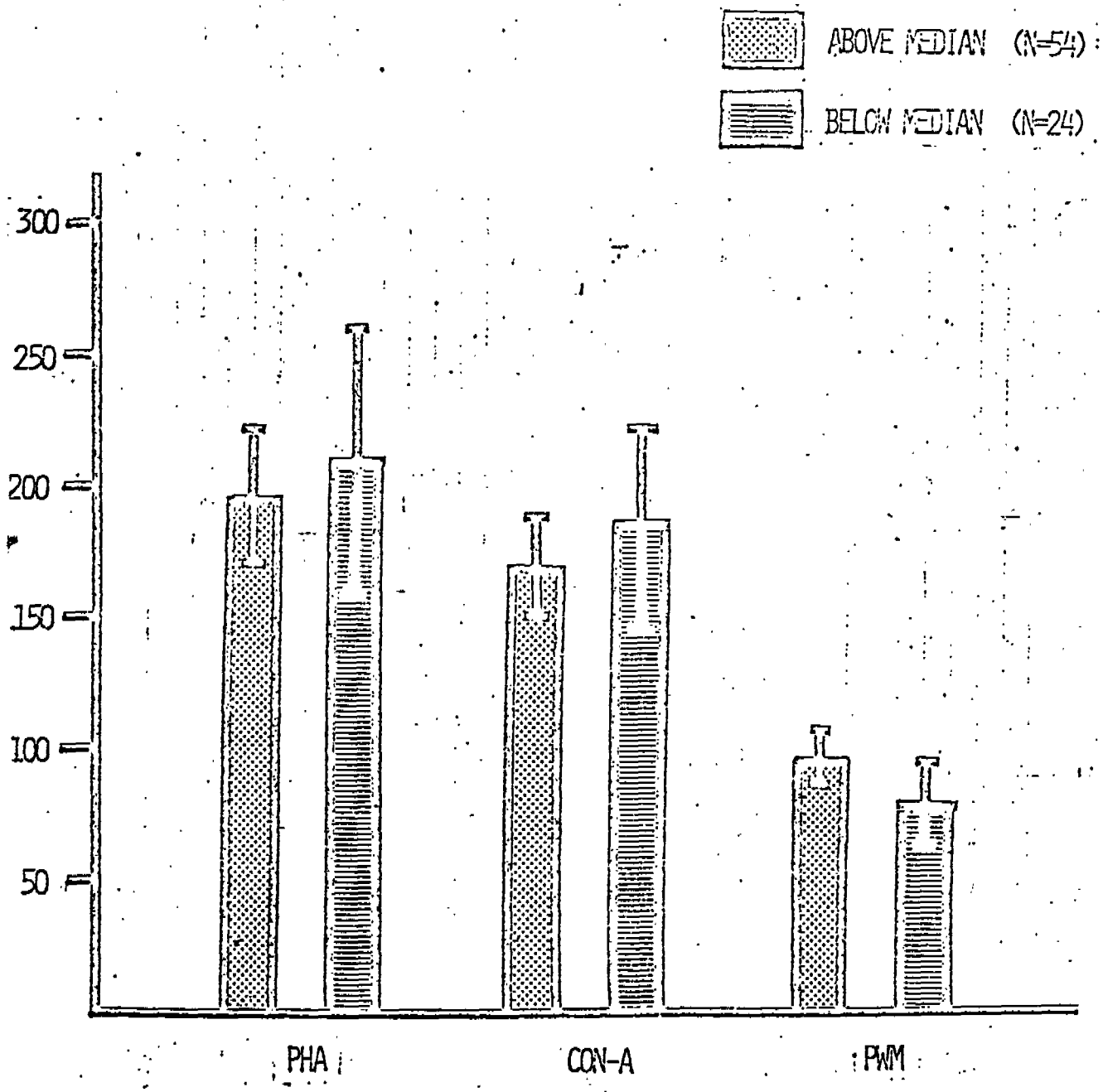

Table 2

ADDITIONAL IMMUNE PARAMETERS OF VC WORKERS ACCORDING TO EXPOSURE

TEST	Above Median Exposure	Below Median Esposure	Statistical Difference Between Groups
SPL Stimulation	191 ± 31 (n= 52)	186 ± 34 (n=24)	None
Absolute Lymphocyte Count	2355 ± 132 (n= 52)	2316 ± 148 (n= 24)	None
SRBC Rosettes, 4°C	1503 ± 101 (n= 50)	1524 ± 132 (n= 24)	None
SRBC Rosettes, 33°C	1227 ± 80 (n=50)	1260 ± 123 (n=24)	None
Recall Stimulation			
SLO	8 ± 2 (n= 53)	24 ± 12 (n= 24)	None
PPD	29 ± 8 (n= 49)	18 ± 6 (n= 23)	None
Candida	11 ± 3 (n= 53)	6 ± 2 (n= 23)	None
Varidase	29 ± 5 (n= 54)	41 ± 12 (n= 23)	None

TISSUE AND URINARY GLYCOSAMINOCLYCANS IN HEPATIC FIBROSIS AND HEPATIC CANCER

Charles E. Kupchella, Ph.D.

Because characteristic glycosaminoglycan (GAG) changes have been found in many kinds of tumors and in tissues undergoing fibrogenesis, and because they have been shown to be present in large amounts in blood vessels, and because tissue GAG changes have been shown to be reflected in urinary excretion in certain disease states, we asked the question: "Are there characteristic glycosaminoglycan changes associated with hepatic angiosarcoma and other chemically-induced diseases of the liver and are they reflected in urinary excretion such that they are exploitable in early detection and/or diagnosis?" To date we have found that:

1. Human hepatic angiosarcoma is accompanied by elevated hepatic GAGs. (1)

2. The GAGs in the angiosarcomatous tumors are different from those in fibrotic tissue adjacent to the tumors. (1)

3. Angiosarcoma and hepatoma patients have characteristic urinary GAG patterns -- patterns not found in workers with non-angiosarcomatous liver injury. (2)

4. Heparan sulfate (a type of GAG) is elevated in hepatic tissue undergoing experimentally induced fibrosis and heparan sulfate is elevated in the urine of such animals.(3)

5. Yet, uncharacterized, chondroitin sulfates and hyaluronic acid levels -- but not heparin -- are 3-4 fold higher in experimentally transplanted hepatomas than in normal liver and urinary excretion reflects both the tumor GAG composition and the size of tumors.

6. Livers of animals bearing metastasizing hepatoma (5123tc) have 50-fold greater concentrations of a non-sulfated, neutral, uronic, acid-positive material than is found in the livers of animals bearing two other, non-metastasizing hepatomas.

Glycosaminoglycans are important in the etiologies of both hepatic fibrosis and hepatic cancer. To date, our work indicates that different GAGs are involved in these processes. We feel that the roles of the GAGs in the hepatic fibrogenesis and as partial determinants of cancer cell behavior are of such importance that GAG-directed strategies may be evolved for the control of these diseases as well as for their detection and diagnosis. (See Slides 1 and 2).

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Subject to Protective Order in
Poss v. Conoco, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

SLIDE 1

Kupchella, et al.

LIST OF STUDIES/EXPERIMENTS COMPLETED TO DATE
AND THOSE IN PROGRESS

1. Evaluate urinary GAG excretion total in 50 human samples -- angiosarcomas, hepatic fibrosis, normal controls, hepatitis, etc.

2. Evaluated human hepatic angiosarcoma, hepatoma cirrhotic liver tissues GAGs (10 cases).

3. Evaluated tissue and urinary GAG patterns in animals undergoing CCl_4-induced fibrosis (48 animals) at 3, 6, and 9 weeks (histochem/biochem).

4. Repeated # 3 evaluating at 1, 2, and 3 weeks (42 animals) histology/ biochemistry.

5. Evaluating urinary and tissue GAG patterns in animals bearing different behavioral types of transplantable hepatomas (42 animals).

6. Evaluating tissue and urinary GAG patterns in animals following partial hepatectomy.

7. Evaluating diurnal variation in urinary GAG excretion in two angiosarcoma cases (vs. normal).

   Compared urinary GAG patterns in workers with known normal and known diseased livers -- 24 workers (complete fractionations using Dowex 1X2 exchange).

8. Compared urinary GAG patterns with other non-invasive tests as to diagnostic value. One hundred-twenty (120) samples (used occasional samples with creatinine determinations).

9. Evaluating the contribution made to urinary GAG excretion by hepatic necrosis (experimentally induced ischemic necrosis in rats).

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NOTE: These are some preliminary da athered in our transplantable hepatoma studies. These studies are designed to evaluate the relationship between tumor GAG composition and various tumor properties (growth rate, metastatic potential, etc.) and to evaluate urinary GAG excretion as a function of tumor size.

TABLE: URONIC ACID LEVELS BY GAG FRACTION IN MORRIS HEPATOMA TISSUE; LIVER OF TUMOR BEARING ANIMALS; AND, NORMAL LIVER

SLIDE 2

	TUMOR 7777	TUMOR 5123	TUMOR 9618	LIVER 7777	LIVER 5123	LIVER 9618	NORMAL LIVER
0.03 M NaCl NON-GAG CARBOHYDRATES	176 ± 38	176 ± 15	524 ± 81	664 ± 203	6770±1629	455 ± 146	950 ± 340
0.4 M NaCl HYALURONIC ACID	90 ± 10	91 ± 12	111 ± 9	40 ± 8	41 ± 5	49 ± 10	25 ± 3
1.2 M NaCl CHONDROITIN SULFATE	261 +29	217 ± 51	208 ± 22	68 ± 23	58 ± 5	41 ± 10	47 ± 7
2.1 M NaCl HEPARIN	31 ± 7	17 ± 4	67 ± 11	17 ± 3	28 ± 6	31 ± 7	39 ± 10

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URINARY GLYCOSAMINOGLYCAN PATTERNS IN ANGIOSARCOMA OF THE LIVER

KEVIN L. CURRAN, BA, MS, CHARLES E. KUPCHELLA, PHD, AND CARLO H. TAMBURRO, MD

Glycosaminoglycans extracted from 24-hour urine specimens from patients with hepatic angiosarcoma and from normal/controls were separated as cetylpyridinium complexes into "hyaluronic acid," "chondroitin sulfate," and "heparin" fractions, then further separated and characterized by anion-exchange chromatography and hyaluronidase susceptibility. The chromatographic pattern of the urinary chondroitin sulfate fraction in patients with angiosarcoma of the liver differed from those of controls in that there was a relative increase in the total amount of uronic acid in a hyaluronidase-resistant fraction and a decrease in a fraction susceptible to hyaluronidase digestion. These changes appeared to become more pronounced with advancing disease. Chromatographic patterns and determinations of hyaluronidase susceptibility indicated that the resistant fraction was heparan sulfate and that the susceptible fraction was chondroitin-4-sulfate and/or chondroitin-6-sulfate.

Cancer 40:3050–3053, 1977.

THE EMERGENCE OF ANGIOSARCOMA OF THE liver and its relationship to vinyl chloride exposure[4,7] has prompted a search for methods to detect this lesion. Although systematic screening programs are currently in operation,[10,16] there is still no single chemical indicator which is specific for angiosarcoma or for changes which may precede this disease.

The association of elevated tissue glycosaminoglycans (GAG) with tumors, including angiosarcoma, has been established.[1,9,15,18,22] Glycosaminoglycans are also known to be involved in normal connective tissue synthesis and collagen deposition and are elevated in connective tissue disorders.[13] Since angiosarcoma of the liver has both neoplasia and fibrogenesis in its etiology[17] GAG changes could be expected to serve to signal the appearance of early lesions and may be useful in evaluating advanced lesions.

Galambos[6] suggested that since the liver contributes very little to the overall connective tissue of the body, hepatic fibrogenesis should not be expected to result in significant increases in urinary GAG or collagen degradative or synthetic products. Preliminary studies in our laboratory, however, demonstrated an increase in both liver and urinary GAG in patients with angiosarcoma, chronic active hepatitis, and cirrhosis.[15] Most of the increase in urinary GAG occurred in the chondroitin sulfate fraction and, in contrast to what was found for normal and other diseases, the urinary chondroitin sulfate fraction was the *only* uronic acid positive fraction found in the urine of seven of nine cases of vinyl-chloride-exposure-associated liver injury other than angiosarcoma. This study was undertaken to characterize more completely the urinary "chondroitin sulfate" fraction in hepatic angiosarcoma.

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CLINICAL SUMMARIES

Case 1—(Hepatic Angiosarcoma—advanced)

A 46-year-old white male worked as a chemical helper in a vinyl chloride polymerization plant for thirteen years prior to the diagnosis of angiosarcoma. Twelve years after initial employment, the patient exhibited a persistent elevation of lactic dehydrogenase and underwent angiographic studies which demonstrated multiple areas of scattered tumor stain throughout both lobes of the liver with areas of central translucency consistent with the diagnosis of angiosarcoma of the liver.

Exploratory laparotomy and liver biopsy confirmed this diagnosis, and the patient was treated with adriamycin, cyclophosphamide, and methotrexate; an

initial response was associated with a decrease in the alkaline phosphatase activity, improvement in indocyanine green clearance and an increase in radioisotopic uptake in areas of previously defective uptake. After completion of the chemotherapy course, hepatic function deteriorated and the patient underwent partial hepatic lobe radiation (total dose of 5,000 rads) over a two-month period. Despite radiation therapy, the clinical course continued to deteriorate with the development of ascites, peripheral edema, increasing jaundice, hypoalbuminemia, and marked elevations of transaminases and alkaline phosphatase activities. This was followed by progressive hepatic failure, hepatorenal syndrome and hepatic coma. Autopsy findings showed extensive involvement of the liver with angiosarcomatous tissue extending into the diaphragm and metastasis to retroperitoneal and mediastinal lymph nodes, lungs, right adrenal gland and cerebellum. The right lobe of the liver demonstrated near elimination of the angiosarcoma, presumably due to the radiation treatment. Urinary GAG assays reported here were made on 24-hour urine specimens collected over the two-week period before death (Fig. 1).

Case 2 (Hepatic Angiosarcoma—moderately advanced)

A 54-year-old vinyl chloride polymerization worker was first employed as a polymerization vat cleaner 28 years prior to the diagnosis of angiosarcoma. Two years prior to diagnosis, the patient had persistent biochemical liver, abnormalities although he was otherwise asymptomatic with a normal liver-spleen scan. Angiographic studies showed peliosis hepatis. A liver biopsy revealed focal sinusoidal dilatation, mild chronic inflammatory reaction with portal fibrosis, Kupffer cell hyperplasia and dysplasia. Subsequent biopsies demonstrated continued sinusoidal dilitation, atypical and dysplastic Kupffer cells with premalignant changes. The peliosis hepatis pattern became more pronounced and multiple radioisotopic defects were evident on liver scan. A repeat biopsy one year after initial biochemical abnormality demonstrated malignant sinusoidal cells. The patient was treated with a combination of adriamycin, cytoxan, and methotrexate with limited clinical and biochemical response. Death was preceded by peripheral edema, ascites, progressive hepatic failure, and coma. The GAG analyses reported here were made 10 and 6 months before death (Fig. 1, [middle]).

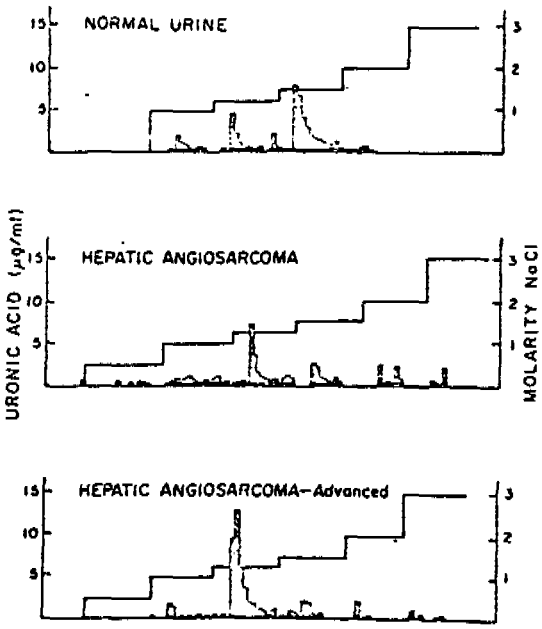

FIG. 1. Elution Patterns of the Urinary Chondroitin Sulfate Fraction. The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("heparin") fractions. Each fraction was then subjected to anion-exchange chromatography. Shown here are typical 1.2 M (chondroitin sulfate) fraction elution patterns (Advanced = case 1).

MATERIALS AND METHODS

Twenty-four hour urine specimens were collected from two patients with angiosarcoma of the liver, and from two normal controls.

Urine specimens were stored at −70°C until analysis. Cetylpyridinium chloride (Sigma Chemical Company, St. Louis) was added to the entire 24-hour volume to precipitate the GAGs according to the method of DiFerrante.[6] The hyaluronic acid, chondroitin sulfate, and heparin fractions were eluted individually according to the method of Schiller *et al.*[19] Cetylpyridinium chloride was removed[14] and the GAGs were subjected to anion-exchange chromatography as described by Schiller *et al.*[19] Glycosaminoglycan fractions were applied to 1.0 × 44 cm AG1-X2 (200–400 mesh, chloride form) columns Bio Rad Laboratories, Richmond, California) and eluted stepwise with 0.0, 0.5, 1.0, 1.25, 1.50, 2.0, and 3.0 M NaCl. At a flow rate of 1.0 ml/min, approximately sixteen 10.3 ml fractions of each molar strength of NaCl were collected and a sample of each fraction was analyzed for uronic acid by the method of Bitter and Muir.[3] Standards of heparin (Nutritional Biochemical Company), chondroitin sulfate (Sigma Chemical Company), and hyaluronic acid (Nutritional Biochemical Company) were also evaluated by ion exchange chromatography.

The uronic-acid-positive fractions within each individual salt fraction were pooled, dialyzed to remove salt, and concentrated. The fractions eluted by 1.25 or 1.50 M NaCl were tested for

TABLE 1.

Source	Ratio of Total Uronic Acid Eluted in 1.25 M/1.5 M NaCl
Normal	0.364
Normal	0.316
Angiosarcoma, case 2, pre-chemotherapy[1]	0.843
Angiosarcoma, case 2, post-chemotherapy[2]	0.971
Angiosarcoma, case 1, advanced	5.000

NOTE: The glycosaminoglycans (GAG) in a 24-hour urine specimen were precipitated with cetylpyridinium chloride (CPC) and separated as 0.4 M NaCl soluble ("hyaluronic acid"), 1.2 M NaCl soluble ("chondroitin sulfate"), and 2.1 M NaCl soluble ("heparin") fractions. The CPC was removed from the 1.2 M NaCl-CPC-solubilized fraction and the GAGs further purified by anion-exchange chromatography. The total amount of GAG in the resulting 1.25 M and 1.50 M NaCl column-eluted fractions was determined and the ratio of the two fractions was calculated. ([1]One day prior to beginning of chemotherapy; [2]Two days following chemotherapy initiation.)

susceptibility to testicular hyaluronidase (Nutritional Biochemical Company) using a modification of the cetyltrimethylammonium-bromide, turbidimetric assay described by DiFerrante.[6]

RESULTS

The major GAG fraction observed in all urines—both from normal controls or from patients with angiosarcoma—was the fraction solubilized by 1.2 M NaCl/1% cetylpyridinium chloride (the "chondroitin sulfate" fraction). Anion exchange chromatography of hyaluronic acid and heparin fractions revealed no qualitative differences between controls and angiosarcoma patients. The anion exchange column patterns of the 1.2 M NaCl solubilized GAGs are shown in Fig. 1. Chromatography of the urinary "chondroitin sulfate" fractions of patients with angiosarcoma yielded a comparatively large, uronic-acid positive peak in 1.25 M NaCl. The ratios of the total amount of uronic acid-positive material eluted with 1.25 M NaCl to the total amount eluted with 1.50 M NaCl are given in Table 1.

TABLE 2. Hyaluronidase Susceptibility

Source	Depolymerization %[1]
Heparin, standard	5.0
Hyaluronic acid, standard	93.1
Chondroitin sulfate, standard	97.6
1.25 M NaCl column-eluate, pooled fractions from angiosarcomatous patients	43.5
1.50 M NaCl column-eluate, pooled fractions from angiosarcomatous patients	100.0
1.50 M NaCl column-eluate, normal	100.0

[1] Glycosaminoglycans isolated from urine were tested for hyaluronidase susceptibility by measuring changes in turbidity developed with the addition of cetyltrimethylammonium bromide following incubation with hyaluronidase. Normal controls exhibited only minor amounts of 1.25 M NaCl column-eluted GAG and consequently do not appear in this table.

The susceptibility of the GAGs eluted with 1.25 or 1.50 M NaCl to hyaluronidase degradation is given in Table 2. The GAG eluted with 1.25 M NaCl was resistant to hyaluronidase, the enzyme producing only a 43% reduction in turbidity. The 1.50 M NaCl-eluted GAG fraction was 100% susceptible to hyaluronidase degradation.

DISCUSSION AND CONCLUSION

The chromatographic pattern found here for controls conforms to urinary glycosaminoglycan distributions reported by others.[12,21] These patterns suggest that there was a relative increase in urinary heparan sulfate and a decrease in chondroitin-4- and/or -6-sulfate in patients with hepatic angiosarcoma. This interpretation agrees with the Dowex 1-X2 chromatographic patterns reported by Kao and Leslie[12] and by others.[2,19]

Heparan sulfate is reported to be partially susceptible to hyaluronidase digestion,[20] and this correlates well with the observed 43% digestion of the GAG in our 1.25 M NaCl fraction. Since heparan sulfate has been shown to be associated with blood vessels,[20] an increase in the urinary excretion of this GAG is not surprising in this vascular lesion. Also, chondroitin-4- and -6 sulfates are reportedly eluted from Dowex 1-X2 columns with 1.50 M NaCl[2,19] and are susceptible to hyaluronidase[20] suggesting that our 1.5 M fraction is chordroitin-4- and/or chondroitin-6-sulfate.

Assuming that urinary GAG patterns described here are reflections of hepatic changes, it will be important to determine what processes these changes reflect, *i.e.*, those of neoplastic growth, fibrogenesis, or cell death. In this regard, it should be noted that 1) the ratio of heparan sulfate to chondroitin sulfate reported here for angiosarcomatous urine is similar to that reported for cirrhotic human liver *tissue* by Becker,[2] and 2) the shift from a hyaluronidase-

susceptible to a hyaluronidase-resistant GAG is consistent with the suggestion by Hutterer and Rubin[11] that the stabilization of collagen depends on a shift to a hyaluronidase-resistant GAG envelope surrounding the collagen bundle. Although Hutterer and Rubin attribute this to an augmentation of dermatan sulfate, Becker[2] reported that the GAG pattern in human cirrhosis was characterized by the augmentation of dermatan sulfate *and* heparan sulfate. If the observed changes in urinary GAG are reflective of vinyl chloride-exposure-associated fibrosis, the fact that fibrosis is a precursor of angiosarcoma[17] indicates that the observations reported here constitute a promising lead in early detection of vinyl-chloride-induced liver disease.

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HEPATIC CELL IDENTIFICATION TECHNIQUE

H.P. FORTWENGLER AND C.H. TAMBURRO, M.D.

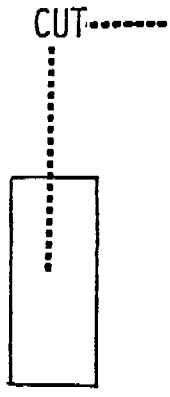
It became very evident early in our vinyl chloride metabolism studies that in order to be able to determine various cell capabilities of oxidizing and detoxifying one needed both an in vivo and in vitro method for determining cell oxidizing and detoxifying capability as well as cell identification. Modification of the technique by Leevy, et al has recently been developed in which the in vitro technique utilizes biopsy tissue for the identification of the various hepatic cell types. This is illustrated in Slide I. A liver biopsy, either human, or animal, is cut in a Y shape in which each of the components of the biopsy can be utilized for different studies; electron microscopy, light electron microscopy and cell type identification. The tissue for cell type identification is then incubated in a modified Eagles medium for two hours at 37°. By incubating this tissue with various markers, such as tritiated thymidine, iron particles, tritiated hydroxyproline or Factor VIII antibody, one can identify cells as being active in incorporation of ^{3}HT into DNA, having a phagocytic capability, having collagen forming ability or simply identies of endothelial cells respectfully. This technique has already been put to use in verifying the cell of origin that malignantly transforms into the angiosarcoma. This has been shown to be the endothelial lining cell and not, as some had thought, the Kupffer or macrophagic cell. This now allows us to direct our attention to the variation in metabolic capability between the primary hepatocyte and the endothelial lining cell. We now hope to be able, using this technique for cell identification, to identify early metabolic capability and morphological changes in cells as they are exposed to various chemicals for prolonged periods of time. This will allow us to determine whether or not these metabolic and morphological effects are progressive, at what stage the metabolic changes are morphologically manifested,

whether these morphological changes are permanent and irreversible, and whether with discontinuance of exposure, these changes revert to normal or progress on to develop malignantcy. We hope that these new developing techniques may be applicable to human tissue via biopsies techniques and help to identify morphological changes with greater specificity as to their etiological cause.

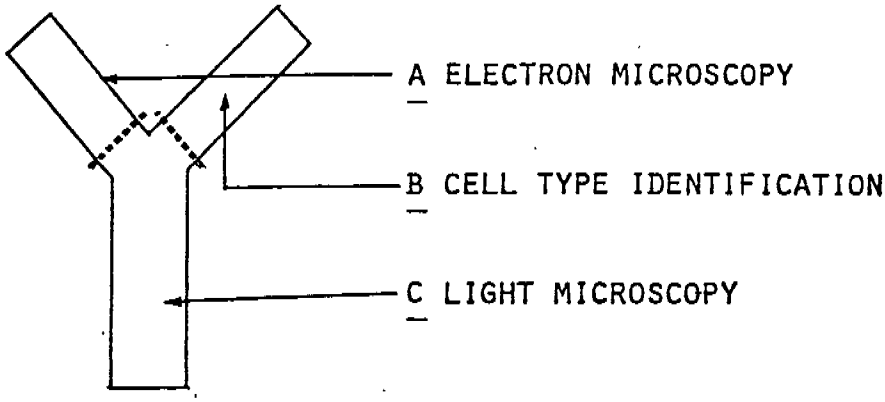
SLIDE I

IN VITRO TECHNIQUE

FOR HEPATIC CELL IDENTIFICATION



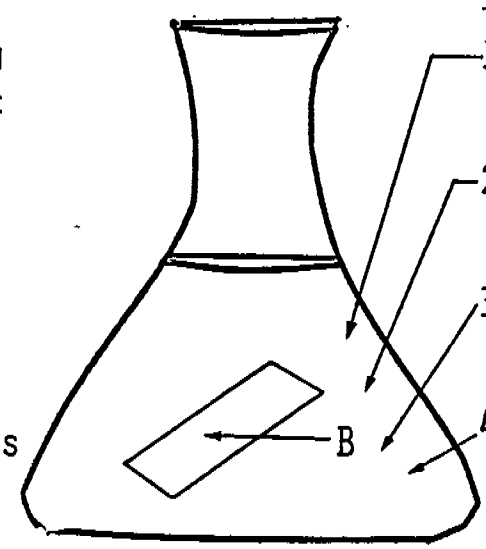
LIVER
BIOPSY



DIVIDED
LIVER
BIOPSY

INCUBATION
2 HR. 37°C

MOD. EAGLES
MEDIA



MARKER	CELL TYPE
1 $^{3}H_{T}/^{3}H_{U}$	DNA/RNA ALL CELLS
2 LATEX/ CARBON/FE	KC
3 FACTOR VIII FLUORESCENCE	EC
4 ^{3}H PROLINE/ VIT A FLUORESCENCE	FB

CMA 002400

MEDICAL SURVEILLANCE SYSTEM FOR INDUSTRIAL ENVIRONMENTS

The University of Louisville has, through cooperative endeavors with St. Anthony's Hospital and the B. F. Goodrich Company, (Louisville Plant) developed a medical industrial surveillance system which is applicable for industry-wide use. The surveillance system is composed of three levels of screening programs as shown in Slide 1.

Level I applies to the industrial environment which does not contain any known or potentially toxic or carcinogenic chemicals. This is also the basis or foundation upon which the more advanced levels of surveillance are built. Level I data base is composed of an employee work history, a job exposure history and a medical illness history including illnesses of the past as well as the present and future.

Level II surveillance is composed of Level I components plus a basic medical history and physical examination, rank ordering of exposure to any industrial chemical thought to be potentially carcinogenic or toxic, and medically and/or Federally required screening tests.

Level III surveillance is designed for an industrial environment actually utilizing a known carcinogen or toxin. At this level a complete medical history and physical examination are conducted. Individual job or person monitoring is done where scientific capability is available; otherwise rank ordering of exposure to the various chemicals is used for each job classification. In addition to Federally required tests, specialized screening studies are performed. A triage protocol is shown in Slide 2, which has been utilized for a combined synthetic rubber and plastic production plant. Slide 3 illustrates the procedure used for medical evaluation of screening data on an individual employee basis. Regional standard range is determined for all laboratory screening tests. Individual tests results outside of the

INDUSTRIAL MEDICAL SURVEILLANCE SYSTEM

THE SCREENING PROGRAM

LEVEL I

A. EMPLOYEE WORK HISTORY

B. JOB EXPOSURE HISTORY

C. MEDICAL ILLNESS HISTORY

(1) INITIAL (PAST)

(2) MORBIDITY (PRESENT)

(3) MORTALITY (FUTURE)

LEVEL II

A. BASIC MEDICAL HISTORY

B. BASIC PHYSICAL EXAMINATION

C. RANK ORDER EXPOSURE - AREA MONITORING

D. BASIC MEDICAL SCREENING

LEVEL III

A. COMPLETE MEDICAL HISTORY

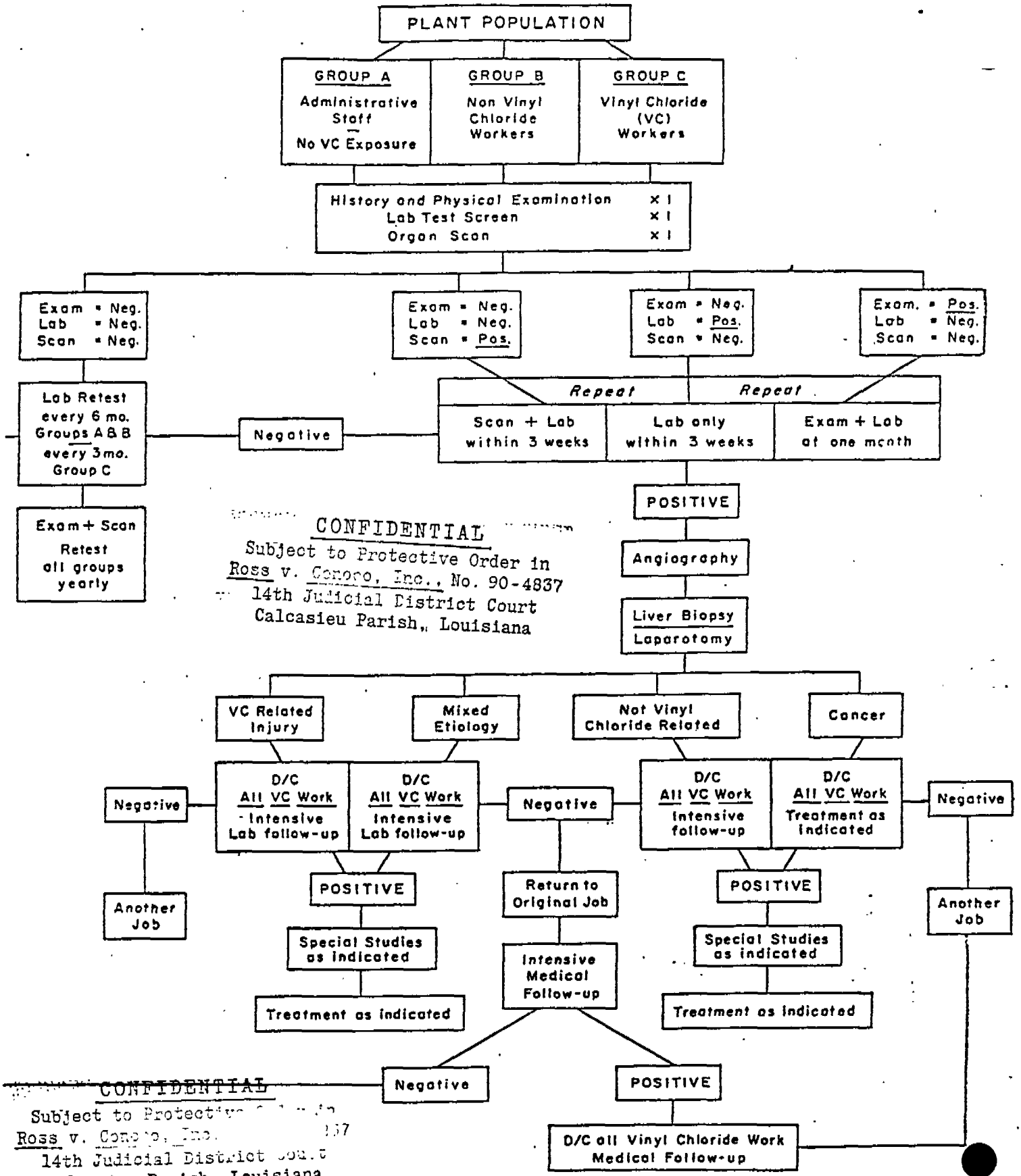
B. COMPLETE PHYSICAL EXAMINATION

C. INDIVIDUAL MONITORING

D. SPECIALIZED MEDICAL SCREENING

SLIDE 2

OUTLINE OF EXISTING PROTOCOL FOR SCREENING



CONFIDENTIAL
Subject to Protective Order in
Ross v. Conoco, Inc., No. 90-4837
14th Judicial District Court
Calcasieu Parish, Louisiana

PROCEDURE FOR EVALUATION OF MEDICAL SCREENING DATA ON INDIVIDUAL BASIS

1. DETERMINATION OF SCREENING TESTS
2. DETERMINATION OF STANDARD RANGE (SR=90%)
3. INDIVIDUAL RESULTS OUTSIDE SR - REPEAT
4. PERSISTENT ABNORMALITY - DIAGNOSTIC WORKUP
5. R - O NATURAL VARIATION - NON-DISEASE
   - EX. (A) AGE - ALK PHOS
   - (B) RACE - IGG
   - (C) GENETIC - INDIRECT BILIRUBIN
6. R - O NON-OCCUPATIONAL DISEASE
   - E.G. (A) HEPATITIS
   - (B) DIABETES
   - (C) HYPERLIPIDEMIA
   - (D) OBESITY
7. INVESTIGATIVE DIAGNOSTIC STUDIES
   - E.G. (A) SPECIAL PROCEDURES
   - (B) HOSPITALIZATION
   - (C) BIOPSY
8. TREATMENT
   - (A) PRIMARY DISEASE
   - (B) OCCUPATIONAL CHANGE

standard range are repeated and individuals with persistent abnormalities undergo diagnostic work-up. There are two major variations in screening tests which may simulate occupationally related disease. These include natural variations, or non-diseases, as exemplified by an elevated alkaline phosphatase associated with age, or elevation of the IgG immunoglobulins associated with race and other areas such as a congenital indirect hyperbilirubinemia which are genetically related. The second category is the non-occupational disease which may simulate occupational injury such as hepatitis, diabetes, hyperlipidemia and obesity. Individuals found to have occupationally-related disease undergo thorough diagnostic studies to determine the etiological cause and are given the appropriate treatment as indicated.

Reasons for surveillance programs are listed in Slide 4. Although the medical surveillance system for industrial environments are designed mainly for the fifth reason, that is, the detection of treatable conditions, the design of this system acutally meets all five reasons for the program, (Slide 4).

BASIC MEDICAL SURVEILLANCE SYSTEM

The employee work history is composed of a uniform job classification code for the entire plant or industry, and estimated exposure (Slide 5). to various chemicals. The degree of exposure is ranked in theorder of 0 to 6.

Slide 6 indicates the rating basis for the rank ordering of chemical exposure. These rank orders are used to develop exposure indices such as average, total, or interaction exposure indices.

Slide 7 illustrates the selection criteria for the original nineteen chemicals used in our medical surveillance system at the B. F. Goodrich Plant. Similar criteria can be used for chemical toxins or potential carcinogens.

Slide 8 is an illustration of a detailed work and exposure history; on the left are listed the years in which the individual was employed, the building code (3 digits), and the job code (an additional 3 digits) and

SLIDE 4

REASONS FOR SCREENING PROGRAMS

1. PROTECTION FOR ECONOMIC BUSINESS
2. PROTECTION FOR OTHER INDIVIDUALS
3. ALTERNATIVE TO PERSONAL HEALTH SERVICES
4. ACQUISITION OF CLINICAL BASELINE INFORMATION
5. DETECTION OF TREATABLE CONDITIONS

SLIDE 5

EXPOSURE SUMMARY

A. JOB CLASSIFICATION CODE
B. EMPLOYEE WORK HISTORY
C. CHEMICAL EXPOSURES
D. EXPOSURE INDICES
   1. AVERAGE EXPOSURE
   2. TOTAL EXPOSURE
   3. INTERACTION EXPOSURE

RATING BASIS

0 - ABSENT FROM ENVIRONMENT

1 - LOWEST EXPOSURE

2 - MINIMAL EXPOSURE TO LOW LEVELS

3 - MODERATE EXPOSURE

4 - WORKS IN AREA SUBJECT TO OCCASIONAL HIGH EXCURSIONS

5 - WORKS IN AREAS WHERE LEVEL IS HIGH

6 - INTIMATE CONTACT - SKIN OR HIGH INHALATION

SELECTION CRITERIA FOR ORIGINAL 19 CHEMICALS

1. IS IT A KNOWN HEPATOTOXIN?

2. IS IT A SUSPECTED CARCINOGEN?

3. HOW TOXIC IS IT?

4. DO EMPLOYEES FREQUENTLY COME INTO INTIMATE CONTACT WITH IT?

5. IS IT FOUND IN THE VINYL CHLORIDE POLYMERIZATION AREA?

DETAILED WORK AND EXPOSURE HISTORY

	WORK HISTORY			EXPOSURE RANK FOR EACH CHEMICAL				
YEAR	BUILDING	JOB	NO. MONTHS	VINYL CHLORIDE	2*	3*	. . . . .	22
1944	000	576	6	2	1	1	. . . . .	4
1945	000	576	5	2	1	1	. . . . .	4
1945	111	194	7	5	4	1	. . . . .	1
1946	111	194	12	6	4	1	. . . . .	1
1947	111	194	12	6	4	3	. . . . .	1
1948	111	194	8	5	4	3	. . . . .	1
1948	121	192	4	4	3	1	. . . . .	1
1949	121	192	4	4	3	1	. . . . .	1
1949	112	253	8	2	6	3	. . . . .	2
.	.	.	.					
.	.	.	.					
.	.	.	.					
1957	121	235	12	4	3	1	. . . . .	1
.	.	.	.					
.	.	.	.					
.	.	.	.					
1972	117	573	5	1	2	1	. . . . .	1
1972	000	574	7	2	1	1	. . . . .	4
1973	000	574	12	2	1	1	. . . . .	4
1974	000	574	8	2	1	1	. . . . .	4
1974	TERMINATED							

* OTHER CHEMICAL

number of months worked at that job in that building. This composes the work history for the employee. The exposure ranking of each chemical is shown on the right. Vinyl chloride exposure is illustrated first and the numbers 2 to 22 are used to indicate the other chemicals.

A multi-employee continuous work and exposure history form for employees is illustrated in Slide 9. This demonstrates a simple means of keeping a work history using a six digit code system for both job classifications and building codes specific for each industry or company. This would provide useful prospective as well as retrospective data to document the safety of the environment or to determine if an occupational risk is truly present.

DATA BANK

The need for a data information resource to allow prospective investigative epidemiological studies have been well illustrated by the University of Louisville's medical surveillance Data Bank System. The material acquired during a three-four year period is illustrated in Slide 10. Packaged computer programs to analyze this occupational medical surveillance data are presently being shown to be cost effective, time saving, able to guarantee uniformity of data collection, as well as security to the workers as far as personal information is concerned (Slide 11). This data, collected on a prospective basis, provides immediately to industry and others, the best available human epidemiological information for validating preliminary results of other industries or animal data. The use of this information for various types of evaluation are shown in Slide 12.

Illustrations of the effectiveness of the program are demonstrated in Slides13, 14, and 15. Slide 13 simply lists the number and types of malignancies found in this cohort of chemical workers between 1960 and 1978. At a glance, one has the impression that there is an increasing number of tumors among liver,

EMPLOYEE WORK AND EXPOSURE HISTORY

YEAR	COMPANY	WORK HISTORY	# OF MONTHS
1945	MILLING, INC.	177* - 268**	14
		177 - 269	26
		178 - 438	20
1950	PLASTIC INTERNATIONAL	013 - 291	24
		018 - 548	12
		013 - 620	48
1957	J.K. MANWORK, INC.	100 - 001	48
		100 - 020	48
1965	G.P. CHEMICAL CO.	001 - 111	CURRENT

* AREA OR BUILDING CODE

** JOB CLASSIFICATION CODE

EXPOSURE DATA FOR EACH CHEMICAL FOR EACH JOB CLASSIFICATION IS KEPT BY THE COMPANY.

SOURCE AND USE OF DATA BANKS:

SOURCE: UNIVERSITY OF LOUISVILLE DATA BANK CONTENTS AS OF MARCH 25, 1977

TYPE OF INFORMATION	NUMBER OF EMPLOYEES
WORK HISTORIES	1672
*EXPOSURES INDICES	1672
BLOOD CHEMISTRIES	1347
LIVER SCANS	1222
SPLEEN SCANS	1217
CHEST X-RAYS	853
HISTORY & PHYSICALS (1974)	679
HISTORY & PHYSICALS (1975)	875
HISTORY & PHYSICALS (1976)	831
BIOPSIES	90

*EXPOSURE INDICES ARE AVAILABLE FOR 21 CHEMICALS IN ADDITION TO VINYL CHLORIDE.

OCCUPATIONAL MEDICAL SURVEILLANCE PACKAGED COMPUTER PROGRAMS

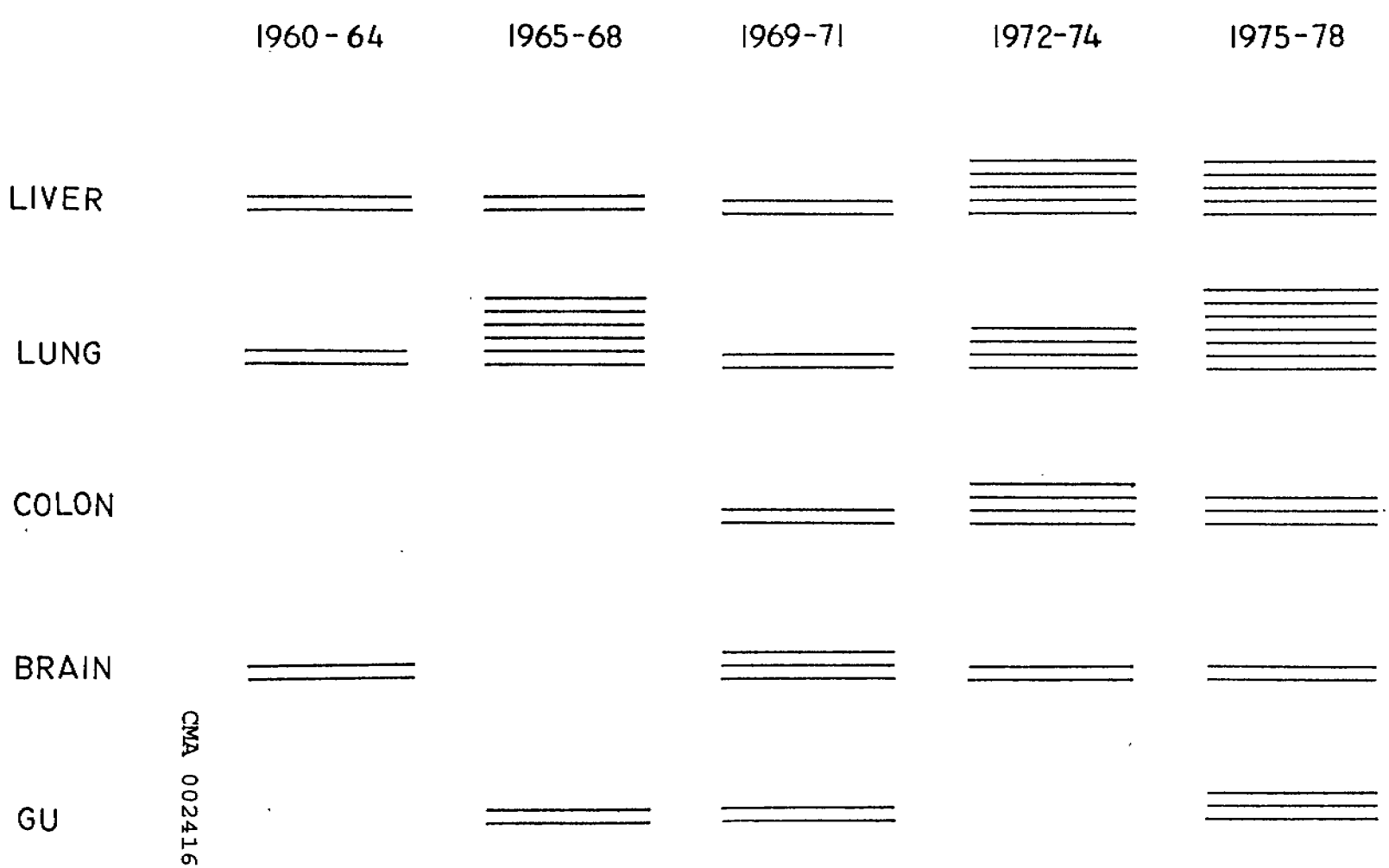
1) WILL SAVE MONEY
2) WILL SAVE TIME
3) WILL GUARANTEE UNIFORM DATA COLLECTION
4) WILL GUARANTEE SECURITY TO THE WORKERS
5) WILL BE AVAILABLE IMMEDIATELY TO MEET THE NEEDS OF INDUSTRY
6) WILL BE AVAILABLE TO BOTH SMALL INDUSTRIES AS WELL AS LARGE INDUSTRIES

lung, and possibly, colon. Analysis of exposure work history helped in identifying correlations between chemicals and cancer. All the cases of angiosarcoma discovered in this cohort population since the start of the program in 1974 were compared with matched controls who had begun to work the same year as the angiosarcoma cases and had worked the same periods of time. Each control and case was then ranked as to their total exposure to vinyl chloride as shown in Slide 14. As one sees, the angiosarcoma cases are among those with the higest exposure to vinyl chloride based on their rank ordering. This was significant to a $P < .05$. In contrast, acrylonitrile (Slide 15) exposure ranking of individuals with angiosarcomas and their match controls shows no such correlation of that chemical to the cases of angiosarcoma. Of the additional 20 chemicals studied, only three others showed significant correlations, as did vinyl chloride, with angiosarcoma. They were a) the specific catalysts for the vinyl chloride polymerization process, b) hexane, used as a solvent for these catalysts, and c) diethyl maleate, a specialized catalyst used only for a specialized vinyl chloride polymerization process showed such relationships (Slide 16). This data validates the ability of rank ordering of exposure to identify causative relationships and/or the absence of such when used retrospectively. This will be even more effective as it is continued to be used prospectively. Additional studies have been conducted to determine if there exists any relationship between lung cancer and vinyl chloride exposure. As shown in Slide 17 and 18, no correlations were found between vinyl chloride, acrylonitrile, or any of the other 21 chemicals when the cases overall lung cancers were matched against their controls. This data would support the lack of a relationship between these chemical exposures and lung cancer development.

EVALUATION

1. ARE SCREENING PROGRAMS TO DETECT DISEASE LIKELY TO HAVE IMPORTANT EFFECTS ON HEALTH?
2. DOES TREATMENT OF RISK FACTORS INFLUENCE DEVELOPMENT OF DISEASE?
3. INDIVIDUAL COMPLIANCE IN SCREENING PROGRAMS,
4. DOES THE SCREENING PROGRAM REALLY ALTER OUTCOME OF TARGET DISEASE?
5. ARE THE PRESENT METHODS OF EVALUATIONS MISLEADING AS TO EFFECTIVENESS OF POSSIBLE SIDE EFFECTS OF SCREENING DISEASE, LABELLING OF INDIVIDUAL AND LONG-TERM THERAPY?

NUMBER AND TYPES OF MALIGNANCY IN CHEMICAL WORKER COHORT—1960-1978



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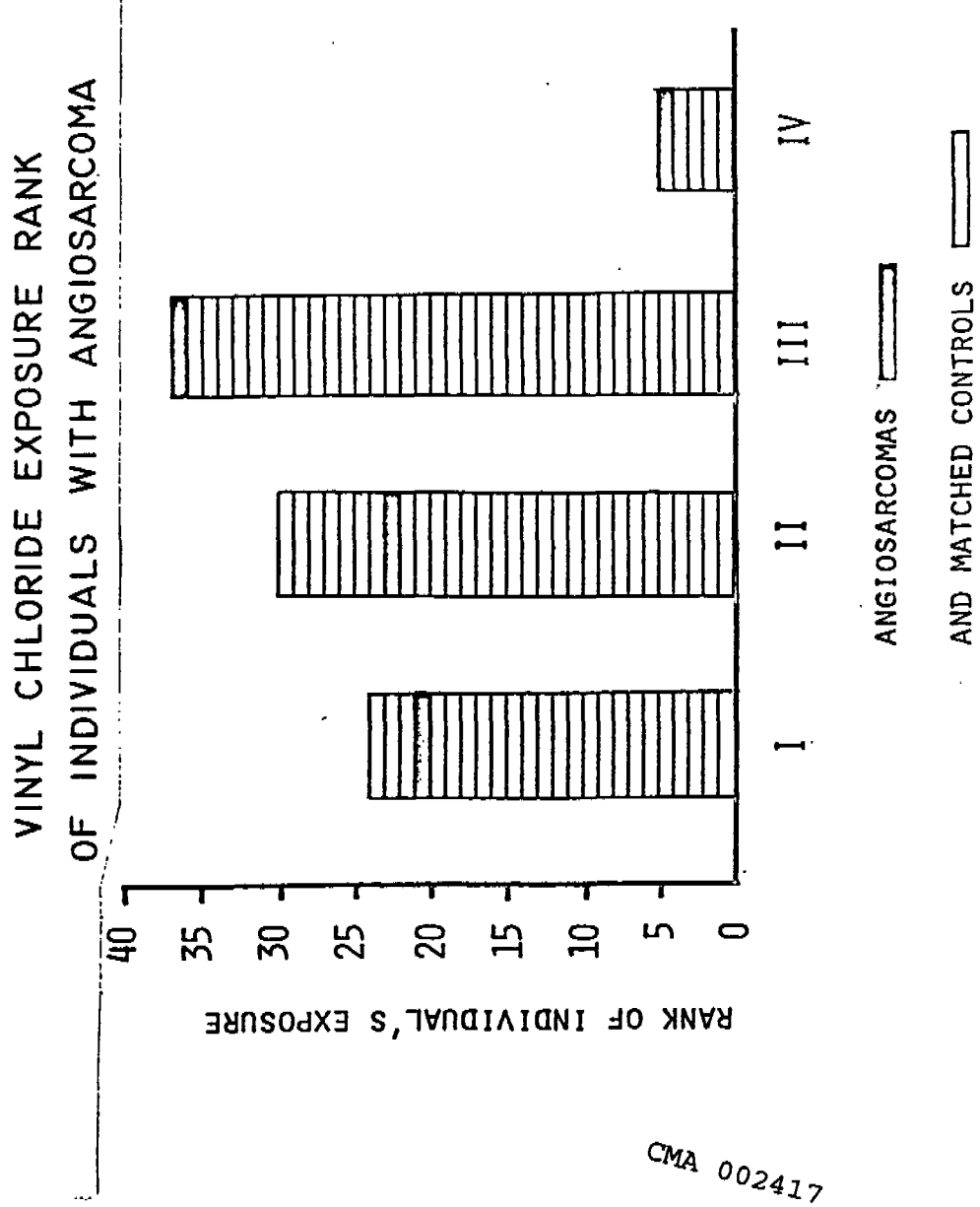

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SLIDE 15

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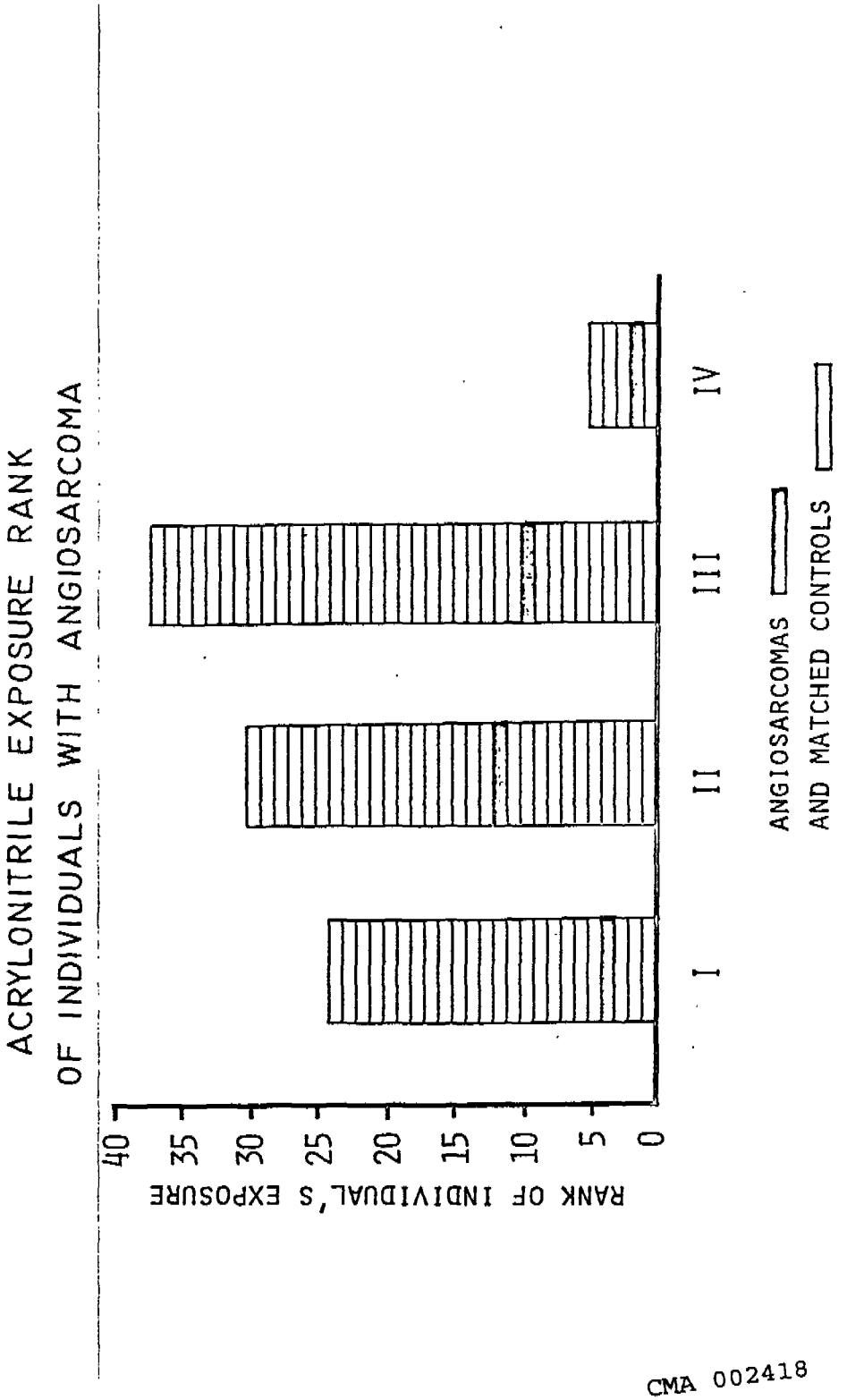

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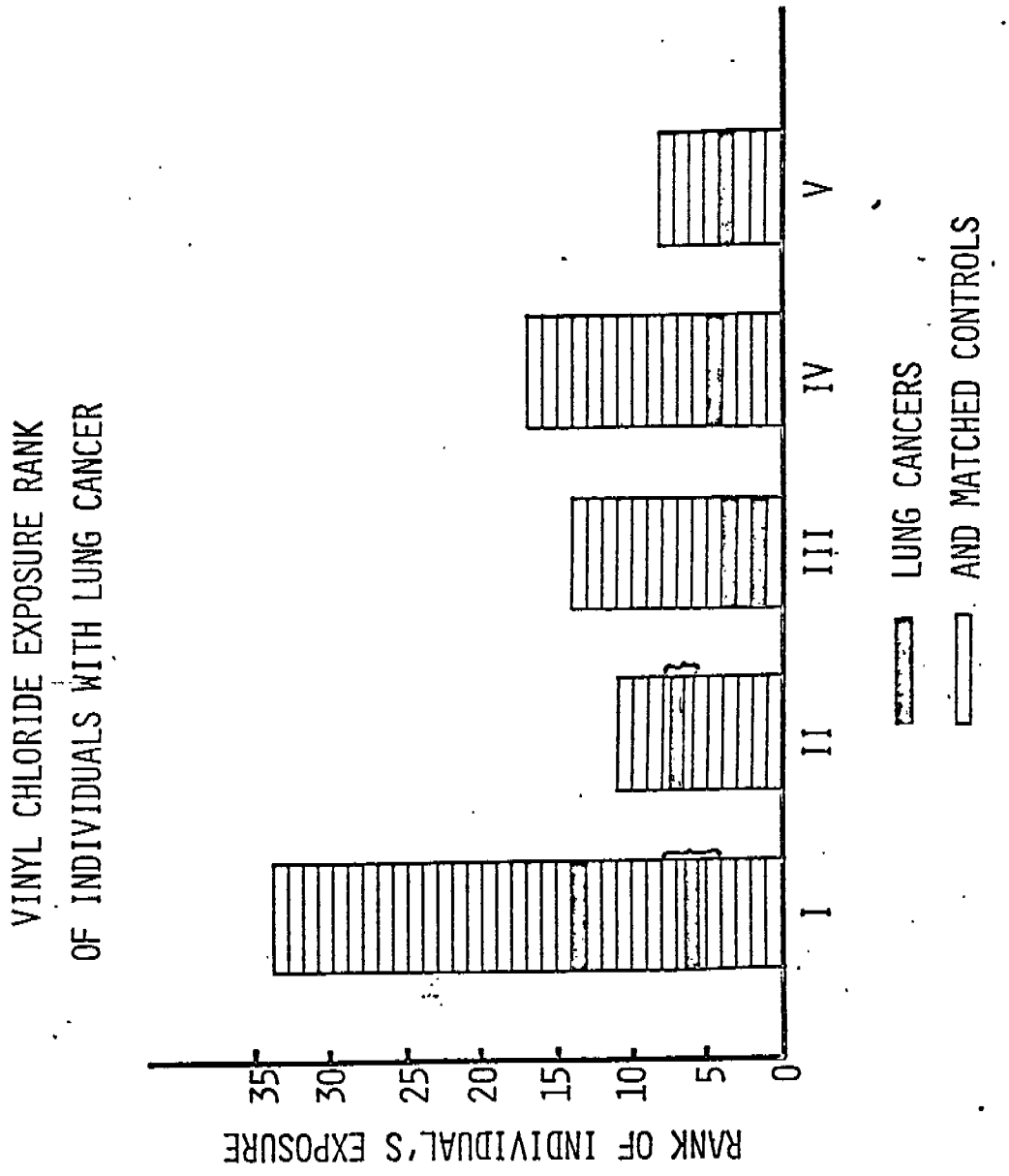
LIST OF SELECTED CHEMICALS FOR EXPOSURE INDICES

CHEMICAL CODE	
01	ACRYLIC ACID
02	ACRYLAMIDES - ACRYLAMIDE, METHYL, N-OCTYL, NMA
03	ACRYLONITRILE
04	ACETYLENE
05	ACRYLATES -- ETHYL, METHYL, METHYL-METH, 2 ETHYL HEXYL, N-BUTYL
06	BISPHENOL A
07	BUTADIENE
08	CAPRYLYL CHLORIDE
09	CHLORINATED SOLVENTS -- CARBON TETRACHLORIDE, CHLOROFORM, TRICHLOROETHYLENE, EDC
10	CHLORO ETHYL VINYL ETHER
11	DIETHYL MALEATE
12	MECURIC CHLORIDE
13	METHANOL
14	PHENOL
15	TOLUENE
16	VINYL CHLORIDE
17	VINYLIDENE CHLORIDE
18	VINYL ACETATE
19	PVC DUST
20	CATALYSTS
21	STYRENE
22	HEXANE



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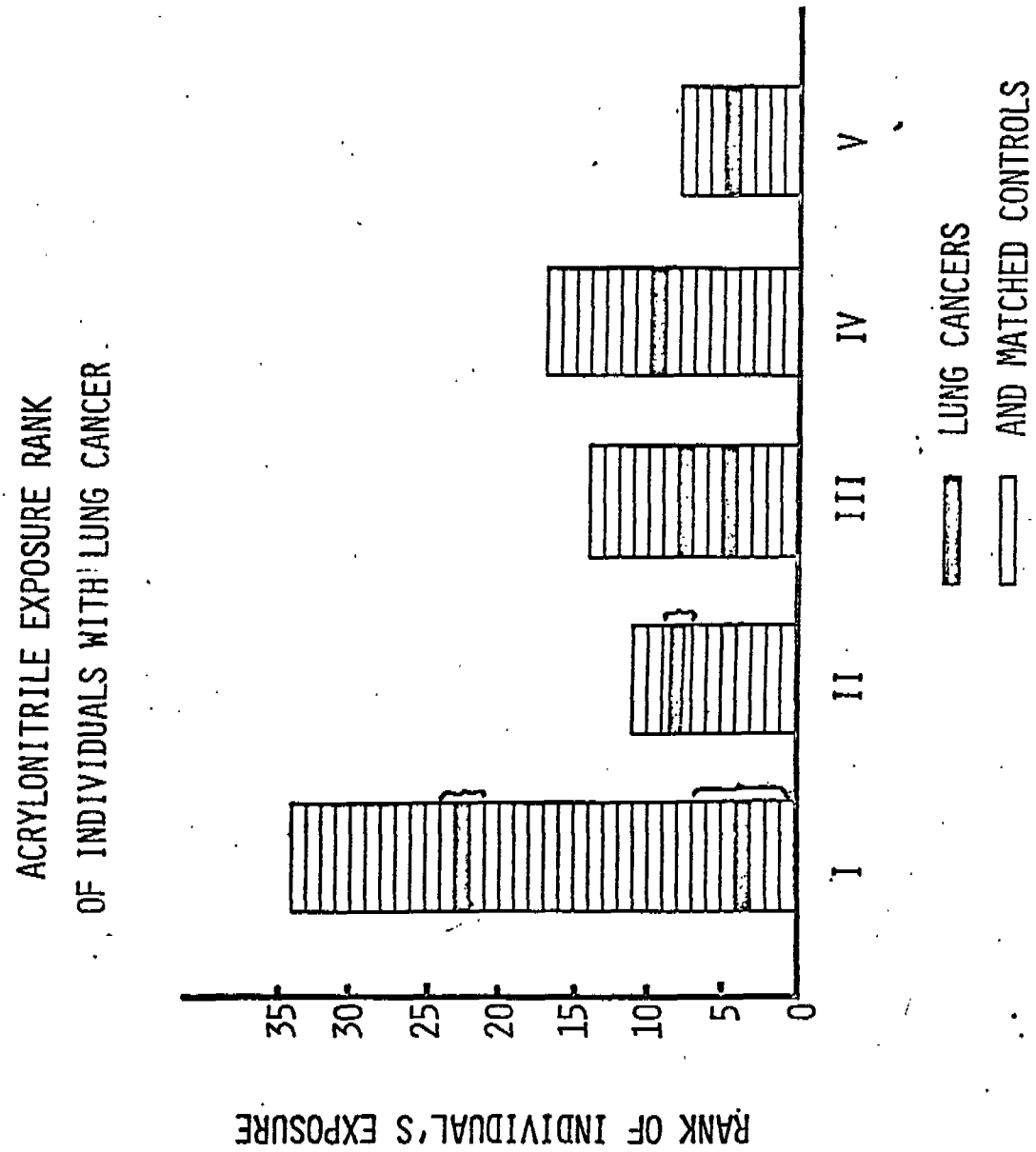


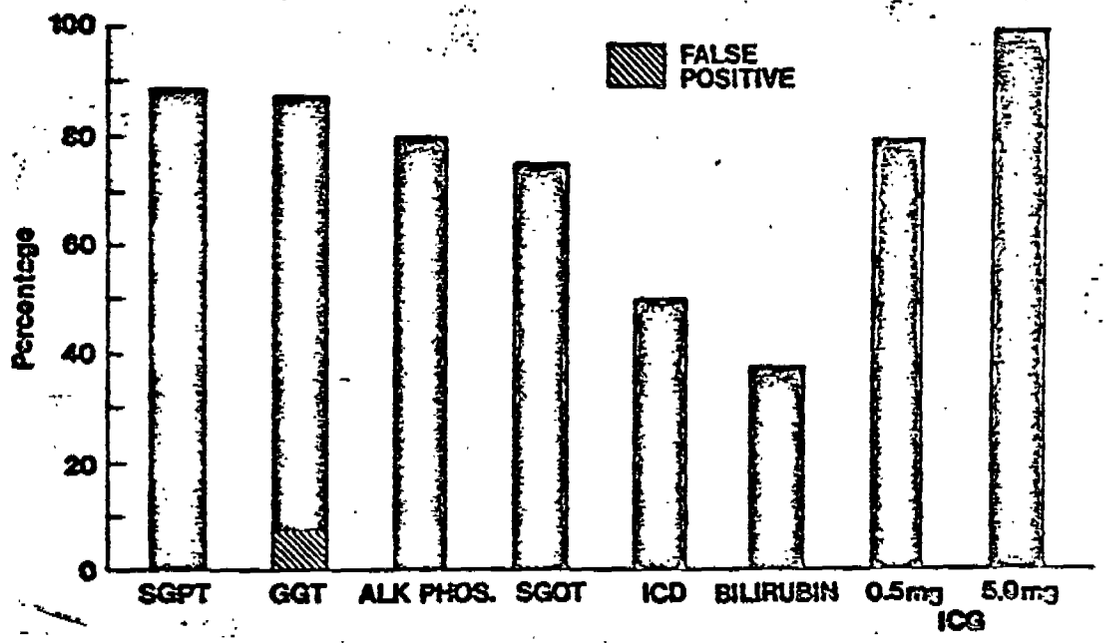
CMA 002421

The use of screening studies to determine the presence of hepatocellular injury have relied mainly on enzymatic studies determined in circulating blood or serum. Slide 19 illustrates the frequency with which the presently available clinical biochemical studies correctly indicated the presence of hepatic injury. The best enzymatic biochemical study was the SGPT which correctly correlated with hepatic injury in about 88% of the cases. The use of functional studies such as dye or bile acid clearances which is shown to the far right, demonstrated that using a maximum dose capacity for the liver, one can correctly identify underlying hepatocellular injury in almost 98% of the cases. The major limitation of clearance studies, however, is the lack of specificity as to the cause of the injury.

In addition, our data indicates that clearance studies are able to demonstrate progressive changes much earlier in the developing of hepatocellular injury than enzymatic studies can. This is illustrated in Slide 20 by the demonstration of progressive, increasing changes in dye clearance in the absence of changes in alkaline phosphatase during progressive exposure to the hepatoxic chemicals over many years. More attention to development of more specific and sensitive single screening procedures are needed rather than a "shot gunapproach" of multiple tests.

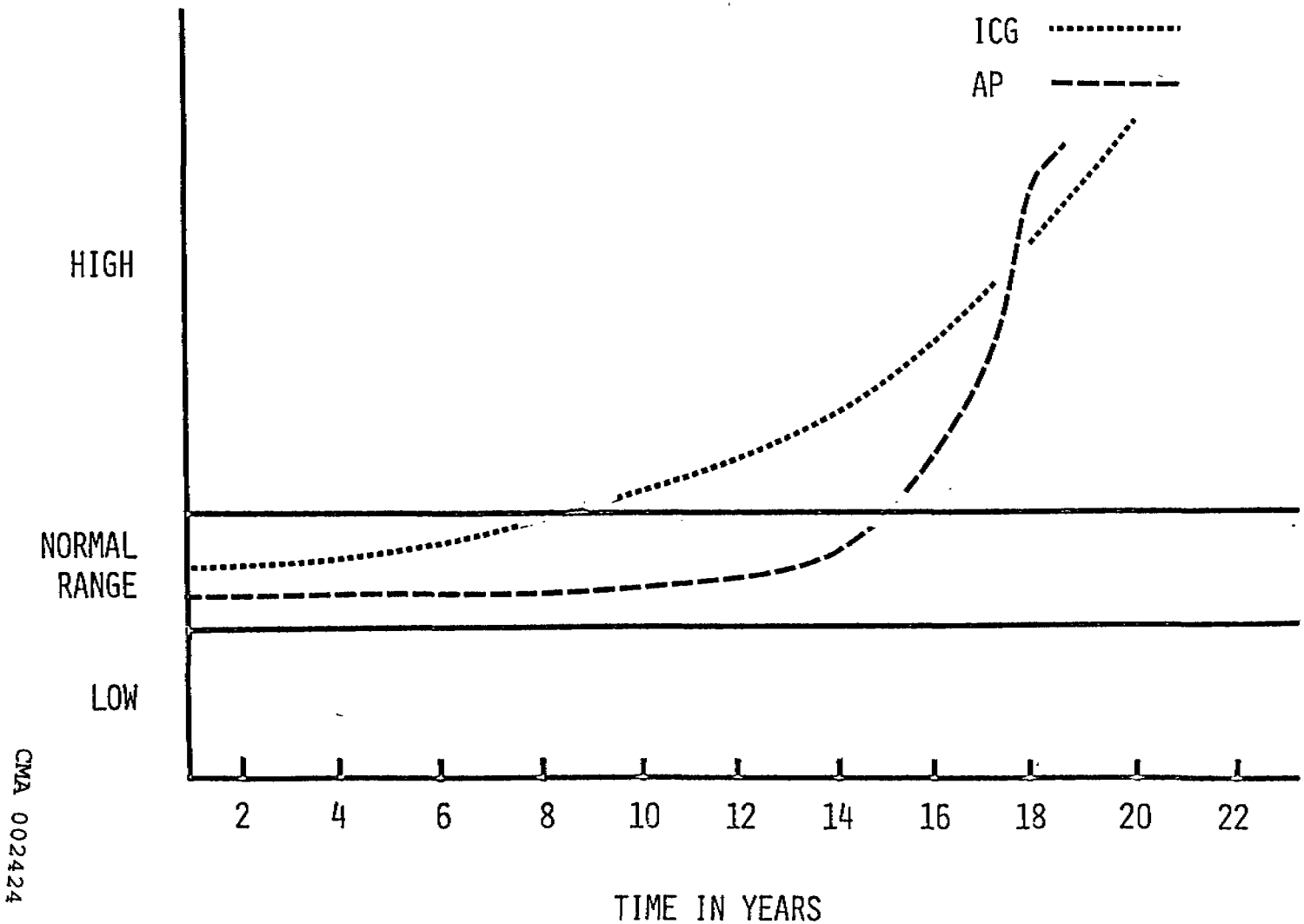
The medical surveillance system is a far more cost effective means of determining industrial environment safety than any presently useable animal or bacteriological screening procedure. Although bacteriological studies are relatively inexpensive, they only indicate which chemicals may be of potential danger. They do not provide any data as to the degree of exposure or duration of exposure needed to present a significant risk.

Animal studies can provide further information, however, the correlation



Frequency of biochemical abnormalities being present and correctly indicating the presence of significant histological abnormalities (SGPT = alanine aminotransferase; GGT = gamma glutamyl transpeptidase; ALK PHOS = Alkaline Phosphatase; SGOT = aspartic aminotransferase; ICD = isocetric dehydrogenase and ICG = indocyanine green clearance)

FUNCTIONAL vs BIOLOGICAL SCREENING FOR HEPATOCELLULAR INJURY IN AN INDUSTRIAL POPULATION



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SLIDE 20

CMA 002424

between developing cancers in animals is not necessarily nor consistently equal to the frequency and occurrence of chemically induced cancers in man. The cost of studying chemical carcinogens (Slide 21) in the four million chemical formulations now available, or the 600 new chemicals reportedly synthesized yearly, or even the 63,000 chemicals estimated to be in common use, are prohibitive in cost. The minimum estimate of cost to study a single chemical used at a single dose level, in a single animal specie for two hundred animals over a two year period, is approximately $40,000. This cost is a conservative one. If this were applied to all 600 new chemicals per year the cost would be $24,000,000. If one were to do these studies solely for the 63,000 chemicals in common use, this would be over $2,000,000,000. In contrast, a medical surveillance system institution in a single plant of 1,000 workers costs roughly $45,000. Once developed, its record maintenance would run about $5,000 per year, or approximately $5.00 per individual. Analyses of the data on a semi-annual, or annual basis, would cost approximately $20,000 for an initial plant and 5-10,000 for each additional plant. This is a relatively inexpensive and provides a much more relevent means of determining environmental safety in a prospective manner. This system can also be utilized to determine and demonstrate whether or not primary preventative methods are effective in reducing the occurrences of cancers in those environments where they are shown to be higher than expected. Such prospective surveillance systems, will provide the means of determining the need and effectiveness of costly preventive requirements which on theoretical grounds are thought to be needed but not proven to be useful. Slide 22 shows how Cancer Centers and Health Science Centers may play an external objective scientific role in assisting corporate medical directors in assessing industrial environmental safety. The corporate medical director responsible for multiple plants working with their part-time or full-time plant physicians can work

SLIDE 21

CHEMICAL CARCINOGENS

4,000,000 CHEMICAL FORMULATIONS

600 NEW CHEMICALS/YEAR

63,000 CHEMICALS IN COMMON USE

1 CHEMICAL, 1 DOSE, 1 SPECIES, 200 ANIMALS,
2 YEARS

COST FOR CHEMICAL TESTING

$2,520,000,000 IN COMMON USE

$24,000,000 FOR NEW CHEMICALS

collaboratively with scientific communities on these problems (Line one). This slide (22) outlines such a working relationship. Various plants dependent on size and need may have, as shown on Line two, no plant physician or full-time (FT) or part-time (PT) one. Line three illustrates various types of plants. A single circle indicates a plant manufacturing a single product (i.e., plastics); multiple circles - multiple products (i.e., plastic and rubbers). The letters below the circles identify the carcinogenic or toxic environment:

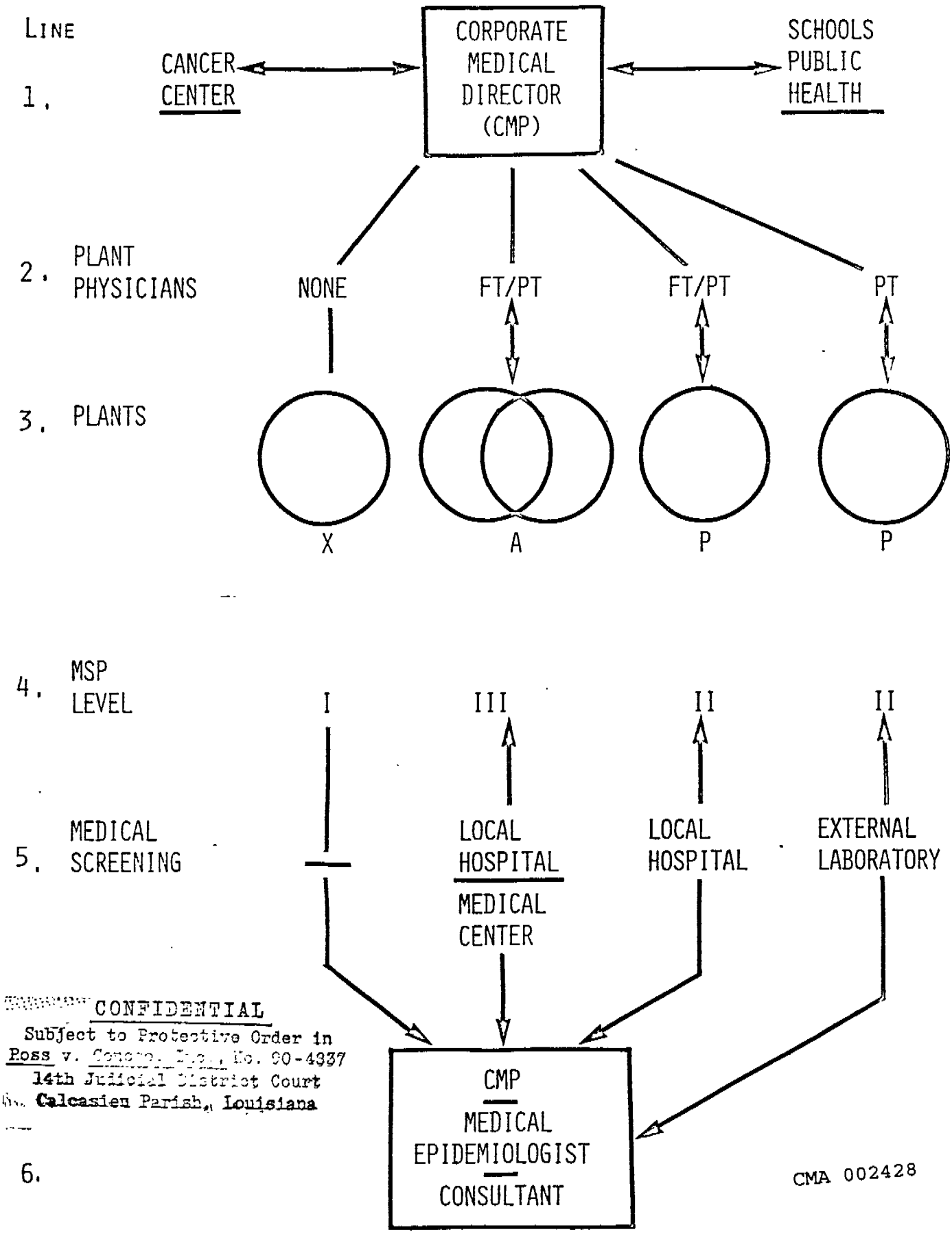
X = no known carcinogen or toxin

A = actual carcinogen or toxin

P = possible carcinogen or toxin

Line four identifies the type of medical surveillance program at each plant. Line five indicates the type of medical screening program being conducted at each plant and Line six indicates the working team of corporate medical director and his staff, Medical Center or Cancer Center medical epidemiologist and medical consultants for prospective ongoing design, review and analysis.

The medical surveillance program's screening studies may be carried on either by local hospitals, or industrial laboratories would supply the test results and medical information to the regional data banks where medical epidemiologists and specialists would analyze the data for the industrial plants in their geographic areas. This information would have the objective scientific status and would prevent claims of biased analysis. If the data were to show problems developing or existing, industry could then apply proper resources to correct them or determine their validity. If the information showed no evidence of developing problems, the data would be utilized to support the safety of the work environment. This system has been successfully used for four years now at the B. F. Goodrich Company, Louisville Plant, in cooperation with local medical facilities, the unions and management. Each component collaborated in the data collection, analysis and validation. Conclusions drawn here have academic, managerial and labor support of their validity and scientific accuracy.



CMA 002428

BIOASSAY OF CHEMICAL MONOMERS IN ISOLATED MAMMALIAN LIVER CELLS

Richard C. Feldhoff, Ph.D. Assistant Professor, Department of Biochemistry, University of Louisville School of Medicine, Louisville, KY 40232

The mammalian liver is the principal organ responsible for the metabolism and/or detoxification of many drugs, hormones and other organic metabolites including potential carcinogens. The exposure of the liver to an hepatotoxin such as ethanol is likely to interfere with its ability to detoxify potential carcinogens. Many of the metabolic and hepatic effects of alcohol have been the subject of extensive investigations, however the concept of the potentiation of carcinogenesis by ethanol is relatively new and has not been adequately investigated. Indeed, recent animal experiments suggest that alcohol intake greatly increases the incidence of cancer when the animals are also exposed to vinyl chloride. In order to better protect industrial workers and the general public from chemically-induced cancers, it appears desirable to define pathways for metabolism and detoxification of chemical monomers by the liver and to assess the role of alcohol consumption on these pathways.

Studies of chemical monomer metabolism can be facilitated by reducing the complexities associated with whole animal experiments to the level of individual liver cells. Recently perfusion techniques have been developed which permit the bonds holding liver cells together to be cleaved when the enzyme collagenase is added to the perfusion medium. The perfusion procedure is depicted in Fig. 1. With the proper perfusion equipment, preparations of isolated liver cells can be readily obtained which exhibit very high viability and have been clearly shown by myself and others to retain normal liver specific functions. An example of some data which I obtained utilizing isolated liver cells is shown in Fig. 2. The synthesis and the secretion of albumin and many other plasma proteins is a specific function of the liver which the isolated cells retain. Additionally, as shown in Fig. 3, the intracellular morphology of the isolated cells when investigated by high resolution electron microscopy is identical to that of the intact tissue.

There are also major technical advantages associated with the use of cell suspensions including 1) the ease of uniform sample collection (media or cells) as a function of time, 2) the ability to assay many different conditions or variables simultaneously and 3) the ability to

employ radioactive metabolites at a much higher specific activity than is feasible in other *in vitro* or *in vivo* systems.

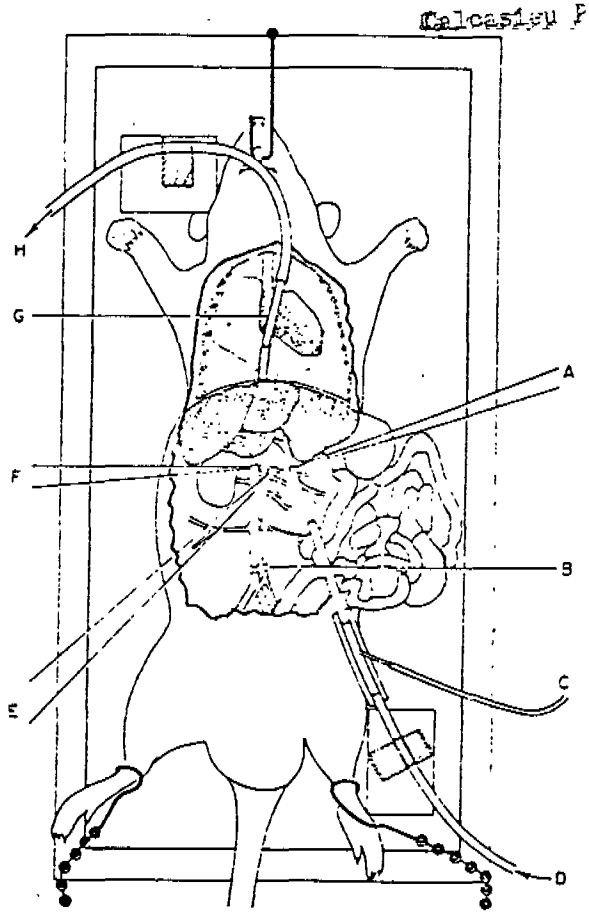


FIG. 1 Diagram depicting cannulation and perfusion procedures. A, ligature tying portal vein cannula and placed above entry of splenic vein; B, inferior vena cava and abdominal aorta sectioned after insertion of portal cannula; C, infusion tubing and cannula inserted into rubber tubing insert in inflow line; D, inflow line taped to aluminum block to hold portal cannula in place; E, ligature around celiac axis and superior mesenteric arteries, tied off after insertion of portal cannula; F, ligature around inferior vena cava above R renal vein, tied off after insertion of vena cava cannula; G, vena cava cannula inserted through right atrium into inferior vena cava, not tied; H, outflow line taped to aluminum block to hold vena cava cannula in place.

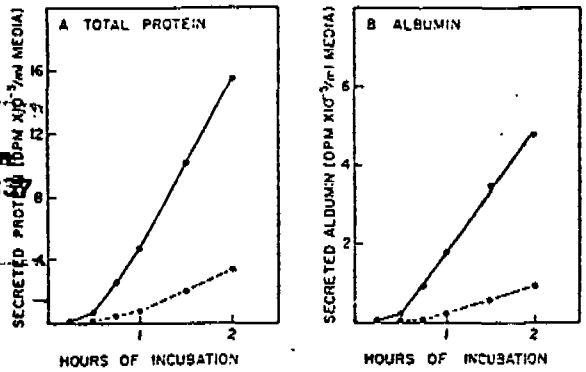


FIG. 2 Total protein and albumin secretion by isolated hepatocytes. Cells were prepared as described under "Experimental Procedures" and preincubated for 30 min in Krebs-Henseleit bicarbonate buffer containing 7.5 times the normal plasma levels of amino acids. Cells were transferred to 40 volumes of bicarbonate buffer containing normal plasma levels of amino acids and [³H]leucine at 10 μCi/ml. At each time point, aliquots of the suspension were centrifuged and assayed for incorporation into total secreted protein and albumin as described under "Experimental Procedures." o——o, hepatocytes from normal rats; o- - -o, hepatocytes from hypophysectomized rats.

With respect to chemical monomer metabolism, isolated liver cells can be used to investigate:

1) the uptake, intracellular transport (binding proteins) and metabolism by various cell fractions of radioactive precursors (Feldhoff, Tamburro, Du, Wong, (Wittliff - cytosol receptors)).

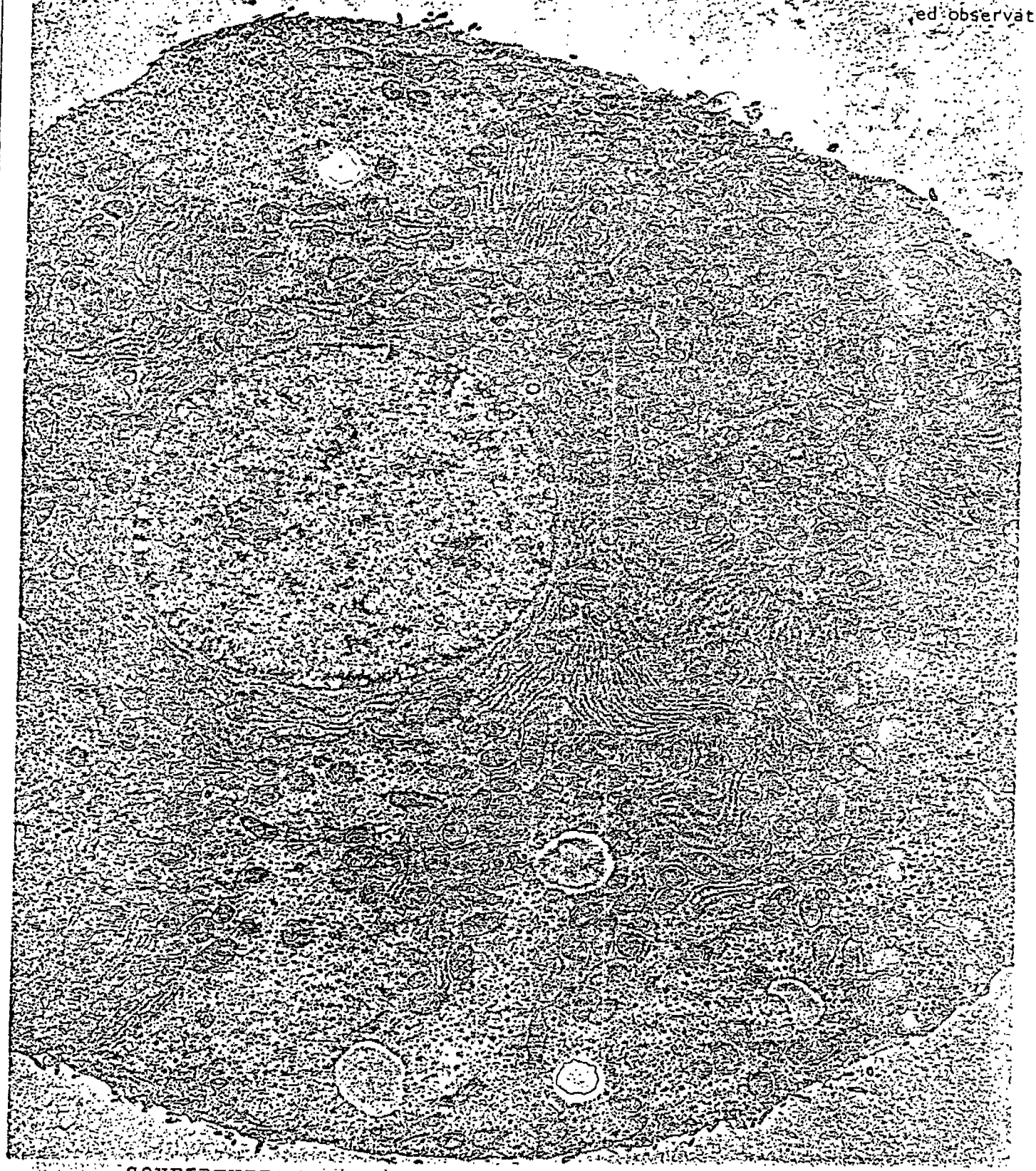
2) identification and quantitation of metabolites released into the medium (Feldhoff, Wong, (Hoffman - HPLC)).

3) effects of individual metabolites on normal parenchymal cell functions (Feldhoff, Tamburro); mutagenic capacities of newly identified compounds (Streips).

4) potentiation of carcinogenesis by concurrent exposure to hepatotoxins such as ethanol (Feldhoff, Tamburro).

This proposed research program would combine the knowledge and experience of the vinyl chloride study group at the University of Louisville with my own research training such that a model system can be developed where individual steps in the metabolism of potential carcinogens can be routinely investigated following acute and chronic exposure. Initially, due to the lack of continuous human liver cell lines these studies would be performed with isolated rat liver cells and extended to human liver cells when they become more available.

Fig. 3 Isolated rat hepatocyte. Cell suspensions were perpared by collagenase perfusion and then incubated for 2 hours at 37° C in Krebs-Henseleit bicarbonate buffer. Utilizing standard procedures the cells were fixed for 1 hour at 23° C in 0.1 M cacodylate buffer, pH 7.4 containing 2.7% glutaraldehyde. Cell preparations were dried in graded ethanol solutions, transferred to propylene oxide and embedded in Epon. The sections were stained wi lead citrate and uranyl acetate. Magnification: 7700X. (Feldhoff, R.C. & Hillman, B., Unpubli ed observati



THE YALE JOURNAL OF BIOLOGY AND MEDICINE 51 (1978), 67-80

The Hepatic Role in Carcinogenesis and Its Early Detection—The Vinyl Chloride Model[1,2]

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Received October 17, 1977

The liver's role in vinyl chloride toxicity and carcinogenicity is providing a better understanding of the chemical carcinogenesis mechanism. A variety of both malignant and benign hepatic tumors has been demonstrated with prolonged exposure to vinyl chloride. The multi-system involvment of this carcinogen and toxin has provided a model for the study of chemical carcinogenesis common to both man and animal. Clinical studies have shown the usefulness of biochemical, radioisotopic, and radiological studies in the detection of toxic and carcinogenic lesions. Animal studies have demonstrated the biochemical metabolism by the liver of vinyl chloride-produced intermediates which are mutagenic in bacterial systems and may be the ultimate carcinogens. Hepatic subcellular enzyme studies prove preliminary evidence of cellular adaptation and increased detoxification. Disruption of this oxidization and detoxification balance may be the key to the malignant transformation of cells. A working hypothesis is presented which may explain the metabolism of vinyl chloride into mutagenic intermediates by the liver cell and the development of malignant transformation by extra hepatic sinusoidal lining cells, lung cells, and brain tissue.

INTRODUCTION

Currently there is a growing concern that chemical compounds may be responsible for most human cancer through environmental contact. Although 100,000 to 200,000 new chemicals are introduced into industry each year, little is known about their effects. These compounds are primarily synthetics and thus not natural to the environment. The view that industrial chemicals may be latent carcinogenic hazards has again been brought into sharp focus by the discovery of vinyl chloride-induced angiosarcoma. Vinyl chloride ($CH_2 = CH-Cl$ monochloroethyene, a gas) is the basic molecule or monomer of polyvinyl chloride and its co-polymers and one of the most important organic intermediates in the plastics industry. The resulting plastic resin, polyvinyl chloride, is used in innumerable consumer and industrial products, such as containers, wrapping film, electrical insulation, pipelines, credit cards, etc. Until recently (early 1970's) vinyl chloride was regarded as being relatively non-toxic [1,2]. Initially this opinion seemed to be supported by the facts that it was used transiently as an anaesthetic and had been used commercially in industry for many years [3].

The direct information on the toxicity of vinyl chloride to man was obtained from experiments by research workers on themselves, from the evaluation of its suit-

[1]Portions of this work were supported by a grant from Manufacturing Chemists Association and by National Cancer Institutes Contract #NO1-CN-55212.

[2]This article is the thirteenth in a series entitled, "Seminars on Liver Disease," that have been presented as part of the Training Program in Liver Disease at the Veterans Administration Hospital, West Haven, Connecticut. Dr. Harold O. Conn, Professor of Medicine, Yale University School of Medicine, and Director of the Training Program in Liver Disease, is guest editor.

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0044-0086/78/5101-0067 $01.40

ability as an anaesthetic [4, 5], and from study of the cases of vinyl chloride poisoning contracted during industrial use [6, 7, 8, 9].

Sporadic studies with vinyl chloride polymerization workers demonstrated varying degrees of hepatic biochemical derangements, hematological abnormalities, and skin changes [9]. Acute short term exposures led to disturbances of the central nervous system, cardiac arrythmias, severe irritation of the mucosal membrane of the eyes and the respiratory tract, and in some cases—severe pulmonary edema with obstruction of the liver and kidneys. Chronic inhalation trials in animals clearly showed that vinyl chloride was toxic to the liver and kidneys as well as irritating to the mucosal membranes and the lungs. Microscopic examination of liver sections showed degeneration of the central lobules while the damage to the kidneys chiefly involved the tubule and the interstitial lining somewhat like that of carbon tetrachloride damage [10, 11, 12].

BACKGROUND

The literature, however, contained virtually no information on damage to man after chronic exposure until Filatova et al. reported disturbances of the blood vessels and nerves in individuals exposed with 20–300 ppm of vinyl chloride on a continuous basis [13]. Cordier et al. [14] and Wilson et al. [15] were the first to report the hitherto unrecognized disorder termed occupational acroosteolysis (AOL) which included the symptoms of tenderness of the fingertips, gradual destruction of bony integrity of the fingers and a Raynaud's-like phenomena.

Studies were initiated in animals to reproduce the acroosteolysis. In 1971, Violi et al. [16] while exposing animals to 30,000 ppm to induce acroosteolysis, accidentally discovered cancer. Maltoni et al. [17] while studying various levels of exposure demonstrated that angiosarcoma occured at 250 ppm; the Manufacturing Chemists Association's studies [18] demonstrated these liver cancers even at 50 ppm. At the same time, Dr. John Creech, at the B.F. Goodrich Chemical Company Plant in Louisville, Kentucky, discovered an hepatic angiosarcoma in one employee. Creech, recalling an earlier hepatic angiosarcoma at the plant, reviewed the medical histories of previous employees. Four additional angiosarcomas were found, further supporting the connection between vinyl chloride exposure and tumor development. Measures to control the levels of vinyl chloride exposure were then instituted following federal regulation.

THE CHEMICAL

Vinyl chloride's chemical structure is a double-bonded, 2-carbon halogenated hydrocarbon which has structural similarities to tri-chloroethylene, an inhalation anaesthetic. As previously noted, vinyl chloride was once considered as an anaesthetic but was discarded because it caused myocardial irritability. Some of its important physical properties include a low boiling point, a high specific gravity, a low solubility in water, and a half-life in air which ranges from 3–20 hours. Knowledge of these physical properties may be important in determining how this agent produces a cumulative effect in the environment which ultimately leads to cancer formation.

Vinyl chloride is both toxic and carcinogenic, as recognized by the wide variety of associated disorders which have been found among vinyl chloride polymerization workers and vinyl chloride-exposed animals. A yet incomplete list of these

TABLE 1
Occupational Vinyl Chloride
Exposure Associated Disorders

1. Thrombocytopenia
2. Reticulocytosis
3. Splenomegaly
4. Hepatic fibrosis
5. Scleroderma-like skin changes
6. Acro-osteolysis
7. Raynaud's phenomenon
8. Leukopenia
9. Hepatomegaly
10. Pulmonary functional impairment
11. Angiosarcoma
12. Cardiac arrhythmia
13. Nephroblastomas*
14. Zymbal gland carcinomas*
15. Large cell lung cancer
16. Brain cancer

*In rats only

associated disorders is shown in Table 1. Animal and epidemiological studies indicate the probability that cancer induction at other sites is also directly attributed to prolonged and excessive vinyl chloride exposure [19].

In order to develop effective methods of prevention, accurate knowledge of the pathogenesis of this environmental chemical in ultimately producing its most destructive effect—cancer—is needed.

EXPERIMENTAL ANIMAL STUDIES—TUMOR FORMATION

The carcinogenicity of vinyl chloride has been demonstrated in a variety of animals as well as in humans at exposure levels that vary from 50–30,000 ppm. As illustrated in Table 2, a variety of tumor types have been found in rats, mice, and hamsters. An extensive list of benign tumors have also been reported in mice, rats, and hamsters exposed to vinyl chloride. Maltoni's group has now demonstrated primary liver cell cancers in exposed newborn rats.

Direct hepatocellular injury as well as pulmonary, mucosal and skin injuries have been shown in directly exposed animals. Pretreatment with many agents increases the toxicity of vinyl chloride; they include phenobarbital, ethanol, polychlorinated biphenyls and pesticides such as hexachlorobenzene [20]. This relationship to vinyl chloride's ability to induce cancer is under study, particularly in view of the industrial environment which allows exposure to many chemicals to occur concurrently.

HUMAN STUDIES

Epidemiological studies in the human strongly suggest that exposure beyond 10 years is associated with increased cancer mortality, mainly digestive system cancers, primarily hepatic [21, 22]. There also appears to be a higher incidence of large cell carcinomas of the lung, brain glioblastoma multiforms, and lymphomas [23, 24], although there is some disagreement as to the interpretation of this aspect of the epidemiological data.

TABLE 2
Carcinogenicity of Vinyl Chloride
(Exposure: 50–10,000 ppm)

Tumor Type	Species
1. Liver—angiosarcoma	Adult humans, rats, mice and hamsters
2. Liver—hepatocellular carcinoma	Newborn rats
3. Lung—adenocarcinoma	Rats and mice
4. Lung—large cell carcinoma	Humans*
5. Mammary adenocarcinoma	Mice
6. Zymbal gland tumors	Rats
7. Nephroblastoma	Rats
8. Osteochondromas	Rats
9. Skin epitheliomas	Hamsters
10. Melanomas	Hamsters
11. *Glioblastoma multiforme*	Humans*
12. Lymphoma	Humans*

*Strongly suggested epidemiologically

The multisystem involvement of this carcinogenic and toxic chemical is further illustrated in man. Early physical findings of vinyl chloride-injury include hepatomegaly, portal hypertension, possible mild systemic pulmonary hypertension, bilateral midzonal pleural thickening of the lung, and splenomegaly with and without increased portal pressure [25]. These anatomical and physiological findings most frequently occur in the absence of the traditional clinical biochemical derangement of the liver. Screening studies of vinyl chloride workers during the past two and a half years have clearly illustrated the irregular and often delayed appearance of abnormalities in aspartate and alanine aminotransferases (SGOT and SGPT), alkaline phosphatase as well as other hepatocellular enzymes. Gamma glutamic transpeptidase (GGTP), believed to be a more sensitive indicator of hepatocellular injury, has proven to have too high a false-positive rate to warrant its use in the screens for hepatocellular injury. Sorbitol dehydrogenase (SDH) studies indicate that this enzyme, which is liver tissue specific, is too insensitive for primary screening but is useful for confirmatory testing.

Anionic dye clearance studies (Indocyanine Green, ICG) have demonstrated the highest specificity and sensitivity of all the primary screening procedures tested when performed at the 5 mg/kg dose level. At the traditional 0.5 mg/kg level, it has the same effectiveness as the aminotransferases and alkaline phosphatase combined. The frequency of abnormal ICG dye clearances increases with prolonged exposure to vinyl chloride as illustrated in Fig. 1 and correlates well with the cumulative exposure to vinyl chloride as well as the histological evidence of hepatocellular injury. These functional studies for detecting hepatocellular injury do not, however, identify the cause.

Clinical studies have illustrated the usefulness of radioisotopic liver-spleen scans as a primary screening procedure for *anatomical* lesions of the liver and spleen. This procedure has proven to be the single most reliable method of tumor detection. Sixteen of the 19 individuals with anatomical lesions were detected by liver-spleen scan. In contrast, only 32 of 950 normal individuals had scan abnormalities which further diagnostic studies proved incorrect. This method provides an 84% sensitivity and 97% specificity, with only a 3% false-positive rate.

Diagnostic angiographic studies of these radioisotopic abnormalities in 80 individuals have demonstrated 3 major lesions. The first is peliosis hepatis, illustrated in Fig. 2. These lesions are usually numerous involving the entire liver, and have a diffuse stain throughout the nodules which persists into the late venous phase without central hypovascularity [26].

A second, similar lesion has been discovered in individuals with splenomegaly, and named lienal peliosis (Fig. 3). These splenic lesions demonstrate a shortened celiac artery to portal vein circulation time, normal portal vein diameters, and increased spleen size. This has been found only in individuals with long-term vinyl chloride exposure.

The final lesion is that of angiosarcoma (Fig. 4). This tumor has characteristic angiographic features of central hypovascularity, midarterial puddling, and a prolonged peripheral tumor stain which continues up to 30–36 seconds after injection. These characteristic findings have allowed differentiation from other primary hepatocellular cancers, benign tumors and benign vascular lesions [26]. These angiographic lesions have been pathologically confirmed with the additional histological finding including peliosis hepatis, sinusoidal dilatation, and activated sinusoidal cells with increased deposits of collagen in the sinusoidal space of Disse. Exploratory wedge biopsies have in addition demonstrated increased subcapsular fibrosis with subcapsular bile duct proliferation plus the often described portal fibrosis [27].

VINYL CHLORIDE METABOLISM AND CARCINOGENESIS

Present biochemical knowledge indicates that vinyl chloride is most likely metabolized by the liver in a three step process [28]. At concentrations less than 50 ppm, vinyl chloride is metabolized by the alcoholic dehydrogenase system into chloroacetaldehyde and monochloroacetic acid.

$$Cl-CH=CH_2 \longrightarrow Cl-CH_2-CH_2-OH \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} Cl-CH_2-CHO \longrightarrow ClCH_2-COOH$$

An alternative pathway which appears to become operative at 220 ppm is oxidation by the peroxidase-catalase system.

$$Cl-CH_2-CH_2-OH \xrightarrow[\text{catalase}]{H_2O_2} ClCH_2-CH_2OOH \longrightarrow ClCH_2-CHO$$

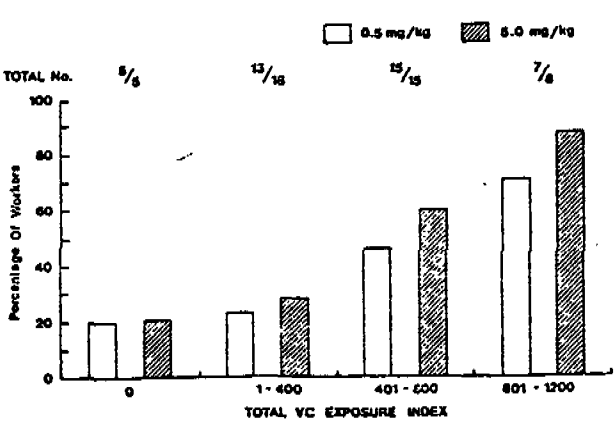


FIG. 1. Frequency of abnormal indocyanine green dye clearance among vinyl chloride workers utilizing 0.5 mg/kg and 5.0 mg/kg doses.

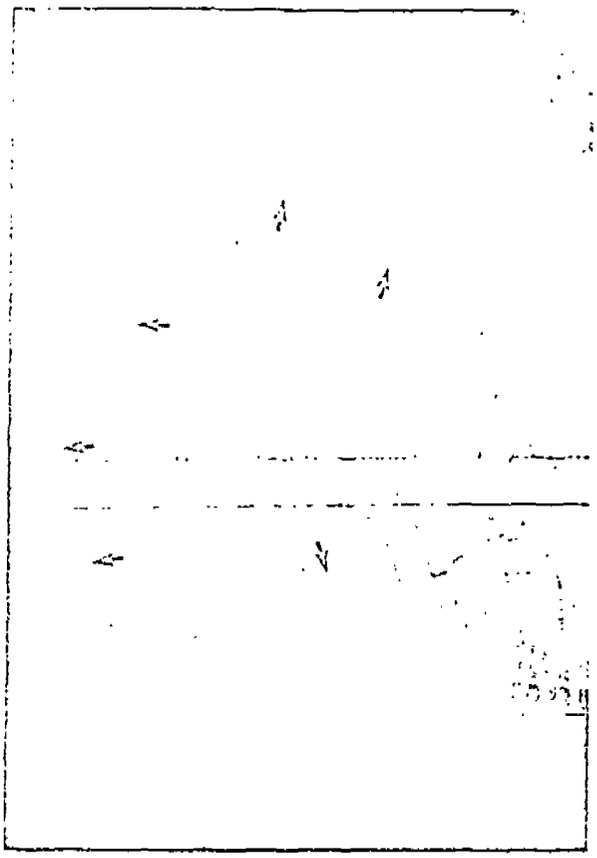


FIG. 2. Hepatic arteriogram: Venous phase. Changes of peliosis hepatis are present throughout the left lobe. The multiple nodular stains represent peliosis hepatis lesions (*arrows*) ranging from 2–3 mm to 2 cm in size.

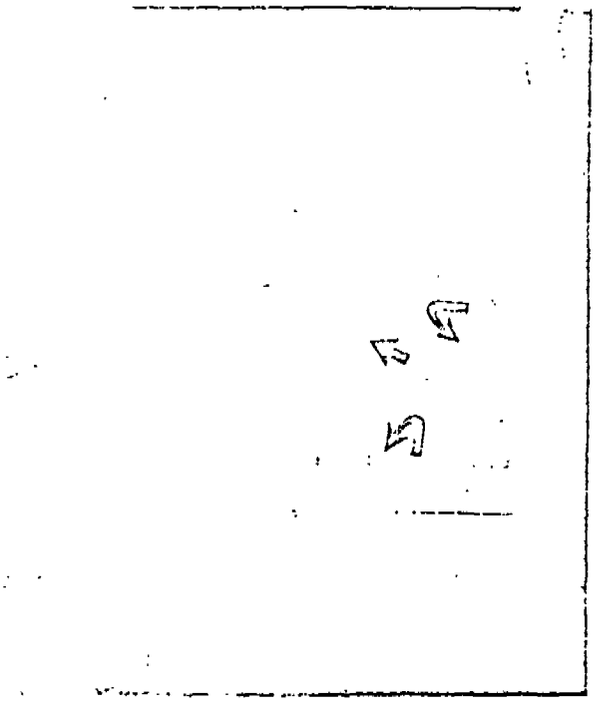


FIG. 3. Splenic arteriogram: Venous phase (15 seconds). There are 3–4 circular and oval stains (*arrows*) in the superior and inferior-lateral portions of the spleen. Normal pancreatic stain occurs just above the splenic vein.

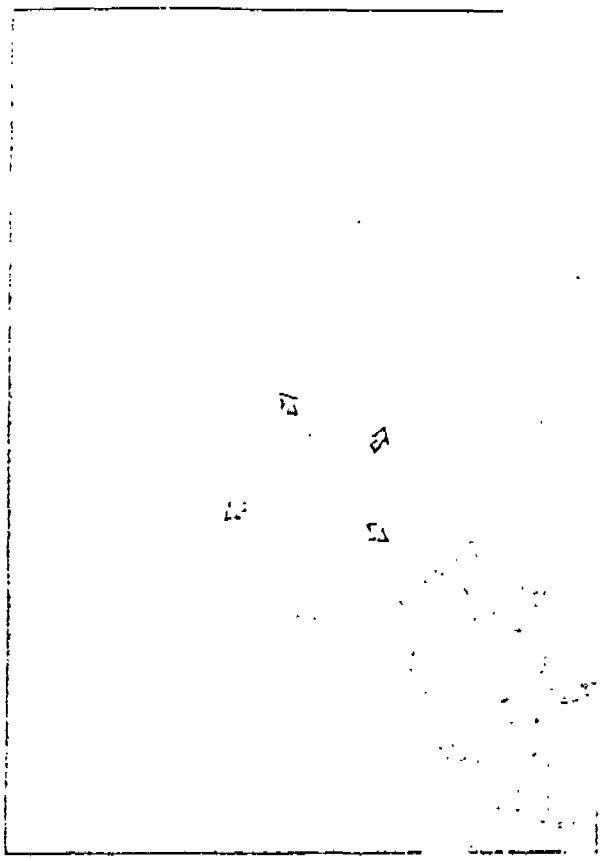


FIG. 4. Hepatic arteriogram: Venous phase. At approximately 14–15 seconds the peripheral stain is identified (*arrows*), lasting through the entire phase. Scattered areas of puddling are also present in and around the area of central hypovascularity.

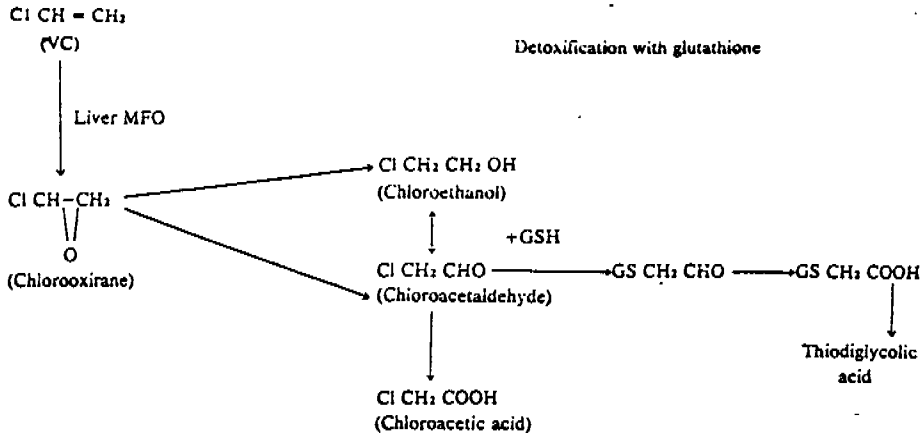
In this case chloroacetaldehyde is again formed. At higher levels oxidation appears to be by the mixed function oxidase system, forming chloroethylene oxide which spontaneously rearranges to form chloroacetaldehyde which then can be further oxidized to form monochloroacetic acid.

$$Cl-CH=CH_2 \xrightarrow{\text{oxidase}} CL-CH\underset{O}{\overset{\diagup\diagdown}{}}CH_2 \longrightarrow Cl-CH_2-CHO \longrightarrow ClCH_2-COOH$$

As illustrated in Table 3, vinyl chloride oxidation intermediates chloroethanol and chloroacetaldehyde, at low doses, are most likely detoxified via the glutathione-cysteine conjugation system. This system, however, is saturable and at higher levels vinyl chloride is excreted via the lungs [28]. It appears that at higher vinyl chloride levels increased amounts of chloroethanol and chloroacetaldehyde are further oxidized to chloroacetic acid which is excreted in the urine. This is further supported by the absence of chloroacetic acid in urines of rats exposed to low, short term levels of vinyl chloride but found in urine of rats exposed to 5,000 ppm for an extended time and reported in workers exposed to levels greater than 250 ppm for a prolonged time [29, 30].

Elmore et al., utilizing a modified Ames system and pure synthesized vinyl chloride intermediates, has demonstrated that vinyl chloride, chloroethanol, and

TABLE 3
Proposed Metabolic Fate of Vinyl Chloride



chloroacetic acid are not mutagenic in bacteriological systems [31]. This may indicate that the vinyl chloride monomer is neither hepatotoxic nor carcinogenic until it has been metabolized to its intermediate forms by the liver and/or other tissues.

Alternatively, chloroethanol—the most transportable of the vinyl chloride metabolites—may be transferred or diffused to adjacent cells, such as the sinusoidal lining cells, where it could be converted to chloroacetaldehyde but less likely to be detoxified or further oxidized. Since vinyl chloride appears to bind the serum albumin, it may, itself be transported to and oxidized by extra-hepatic cells which are unable to fully oxidize or completely detoxify its metabolites, and thereby lead to molecular DNA injury and cancer formation at distant tissue sites.

These quandaries led to further study of the hepatochemical changes in rats undergoing progressively increased exposure to vinyl chloride. Subcellular enzymes and metabolites were studied in animals exposed to from 10–20,000 ppm vinyl chloride, ranging from 14–137 hours. Microsomal enzymes, including P-450, NADPH cytochrome *c* reductase, and mixed function oxidases were studied. Determinations of cytochrome *c* oxidase as the mitochondrial, tritiated-leucine incorporation as the protein synthesis and glucose-6-phosphatase as the carbohydrate metabolism markers were also done. Glutathione and glutathione reductase as oxidative and detoxification markers were determined in addition to the conventional clinical biochemical studies which included the aspartate (SGOT) and alanine aminotransferase (SGPT), alkaline phosphatase, bilirubin, lactic acid dehydrogenase (LDH), total protein, albumin, cholesterol, and triglycerides.

During the entire 137 hours of exposure there were no significant changes in the mitochondrial and the microsomal enzymes, the tritiated-leucine incorporation, or in the glutathione content. There was however, after 71 hours, a rise in the glutathione reductase and a concomitant fall in glucose-6-phosphate. This occurred without any histologically discernible changes in the hepatocytes by light microscopy nor any significant changes in the conventional clinical biochemical studies.

The discovery of a decreased glucose-6-phosphatase after "simulated" chronic exposure led to the study of enzymes in the pentose phosphate shunt pathway. Weber and Lea [32] had found similar changes for primary hepatocellular neoplasms, demonstrating that in a rapidly developing primary hepatocellular tumor, there is decreased gluconeogenesis with a reduction in the glucose-6-phosphatase, followed by an increase in glucose-6-phosphate dehydrogenase and transaldolase. These biochemical changes were also followed by an increase in purine biosynthesis (increased phosphoribosylpyrophosphate aminotransferase (PRPP) and increases in the production of ATP and GTP leading to increased nucleic acid synthesis.

Vinyl chloride-exposed animals showed no significant changes in the glucose-6-phosphatase dehydrogenase activity during the initial 84 hours of exposure. However, after 103 hours, there was significant increase in glucose-6-phosphatase dehydrogenase. Studies of PRPP, at least up to 137 hours, have as yet shown no significant changes. Studies are now underway using animals exposed to 130 to 250 hours to determine if the biochemistry in vinyl chloride injury is similar to that in primary hepatocellular tumors.

This, however, does not explain why the hepatocyte, which is the primary cell for oxidizing and detoxifying vinyl chloride, is not the primary target for cancer transformation. How do the hepatocytic biochemical changes, seen in the early phase of high vinyl chloride exposure, relate to the later morphological changes that occur in the adjacent sinusoidal cells?

MORPHOLOGICAL FINDINGS

Electron microscopic examination of liver sections of mice exposed from 1 to 6 months to 2,500–6,000 ppm vinyl chloride, for 5 hours/day, 5 days/week—a level known to induce angiosarcoma [33]—have demonstrated hepatocellular changes as early as one month. These changes included hypertrophy of the smooth endoplasmic reticulum (believed to reflect vinyl chloride metabolism) and, plasma membrane loss of microvilli with invaginations—possibly reflecting the movement of injurious metabolites across the membrane and out of the cell, allowing the metabolites to be picked up by the sinusoidal cells [34].

The sinusoidal cell reactions were multicellular. Increasing numbers and sizes of lipocytes were seen with little fibrosis. Macrophages were seen filled with phagosomes, sometimes containing long needle-like crystals. Although there were many mononuclear cells present, the main abnormalities were seen in the endothelial lining cells. In the early stages they are larger and thicker—possibly swollen. Later, they became bulky and in places, multi-layered containing increased organelles, especially mitochondria and endoplasmic reticulum. Later disruptions in the sinusoidal walls seen were consistent with beginning peliosis hepatis. The lining cells, probably the precursors of angiosarcoma, often resembled fibroblasts. However, their endoplasmic reticulum did not contain any collagen components. These observations by Schaffner et al. [34] give support to the suggestion that metabolites of vinyl chloride produced in the hepatocytes may be transported through the plasma membrane and enter sinusoidal lining cells, eventually leading to angiosarcoma. Attempts at screening for vinyl chloride hepatic injury might be better aimed at the endothelial cells and the hepatic sinusoidal circulation rather than the hepatocytes.

Our work in humans has identified similar findings. One major difference at

present is an increased collagen deposition, characteristic of human vinyl chloride injury and likely species specific. Light microscopic studies utilizing special stains on hepatic tissue from individuals with extensive exposure to vinyl chloride but without clinical biochemical hepatic abnormalities have shown distinctive midzonal increased deposition of collagen in the space of Disse [35]. Routine light microscopic studies using hematoxylin and eosin failed to easily demonstrate this midzonal increased collagen. The increased deposition along the hepatic cell surface is associated with larger sinusoidal space and activation of the sinusoidal lining cells illustrated by increased nuclear size and cytoplasmic content. The increased collagen deposition, when studied electron microscopically, demonstrates compression of the hepatocytes by the collagen bundles which initially give the appearance of *intra*-hepatocellular collagen bundles as illustrated in Fig. 5. The strands of collagen appear to compress the hepatocytes causing cords of hepatocytes to be broken and to coalesce with adjoining sinusoids eventually leading to peliosis hepatis-like lesions.

These observations led us to the study of the proteroglycan role in collagen formation in vinyl chloride-exposed workers. It had been suggested in the literature that glycosaminoglycans in blood and/or urine might be useful as means of early cancer detection since a number of studies had demonstrated the production of sulfated glycosaminoglycans with malignant states. Pathologists have often used this feature as a diagnostic aid in characterizing malignant vascular tumors of the skin

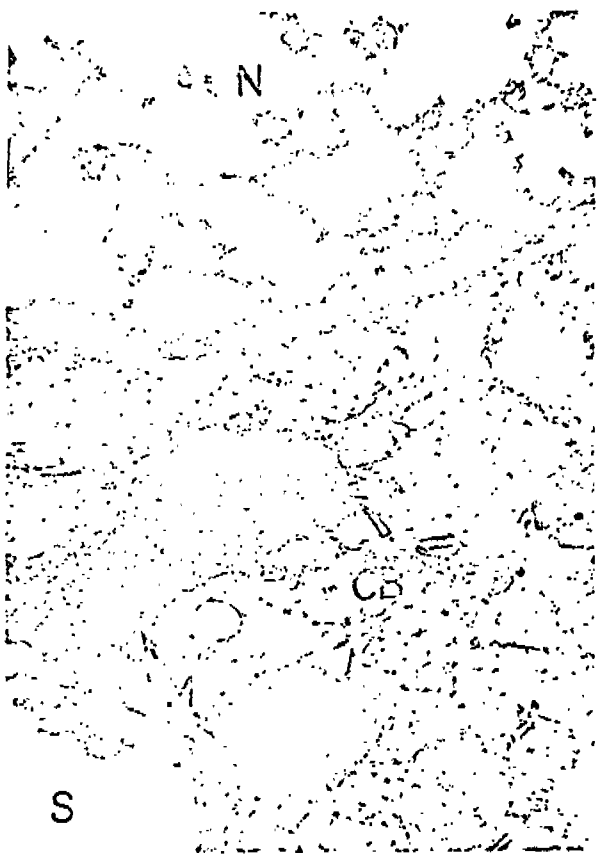


FIG. 5. Electron microscopy showing collagen (CB) bundles (*arrows*) invaginating into the hepatocyte, giving the appearance of inter-hepatocytic collagen. N = nucleus; S = sinusoidal space: IM = invagination into the cell membrane (*small arrows*).

[36]. Others have noted a strong positive Alcian blue glycosaminoglycan staining reaction in human angiosarcoma tissue [37]. This suggested that quantitative and qualitative determinations of glycosaminoglycan production in individuals with neoplasm, either by serum or urine, might be used to identify those at high risk or as an early indicator of neoplastic formation. The feasibility of glycosaminoglycan "spot test" for vinyl chloride production workers made this an attractive possibility for mass screening.

Urinary glycosaminoglycans, measured as uronic acid, were studied in individuals with alcoholic cirrhosis, viral hepatitis, secondary liver metastasis, hepatic angiosarcoma and normal controls.

The percentage of total glycosaminoglycans that was dialyzable and the percentage of unfractionated total that appeared in the hyaluronic acid, chondroitin sulfate, or in the heparin fractions was similar for all groups. However the distribution of positive fractions varied with the different groups studied.

Seven of the nine vinyl chloride-exposed individuals, other than those with angiosarcoma, had glycosaminoglycan positive chondroitin sulfate fractions with negative hyaluronic acid and heparin fractions whereas this occurred in only 3 of the 32 urines from other hepatic diseases [38].

In addition, study of the total tissue glycosaminoglycan levels in angiosarcoma tumors and fibrotic tissue adjacent to the tumor demonstrated that tumor tissue itself had higher levels of hyaluronic acid and heparin fractions as compared to the non-tumor adjacent tissue which had higher levels of chondroitin sulfate fractions. A similar relationship was found in cirrhotic liver tissue and normal controls. This data conforms to reports by others that both hepatic connective tissue disorder [39, 40, 41, 42, 43] and hepatic cancer [44] result in increased hepatic glycosaminoglycan levels. It may be significant that the angiosarcoma patient has half the urinary glycosaminoglycan excretion of patients with liver metastasis and that analysis of angiosarcoma tumor tissue exhibits half the glycosaminoglycan content reported by Kojima et al. [44] for hepatocellular carcinoma. The increases in liver and urinary glycosaminoglycans may well reflect the importance of these substances in the process of fibrogenesis and tumor growth. Although no significant differences were found in total glycosaminoglycans of vinyl chloride-exposed individuals with associated liver injury, there was a significant difference in the excretion patterns of these individuals. Seventy-eight percent of them had positive chondroitin sulfate fractions in contrast to only nine percent of the non-exposed liver injury cases. Thus the change in the glycosaminoglycan excretion pattern in individuals with pre-cancerous injuries may be of significant prognostic and diagnostic importance [45].

The urinary glycosaminoglycan excretion patterns in an angiosarcoma patient 3 months to 2 weeks prior to death demonstrated an increase in the urinary chondroitin sulfate fraction with a change in its composition as the disease progressed. During this time, the chondroitin sulfate composition showed a continuous increase in the ratio of the 1.25 M NaCl to the 1.5 M NaCl fractions. This was due to an increase in the 1.25 M eluate and a decrease in the 1.5 M eluate fraction and was 2.3 times greater than the controls. In the most advanced stage of the angiosarcoma the ratio increased to 13.7 times greater [46].

These very preliminary studies would suggest that alterations in the ratios of these fractions' compositions may be useful in evaluating the severity and subsequent progression of disease. Early lesions may produce small changes in the ratio which would become more pronounced as the disease worsened. The

determination of this ratio might also be useful in identifying those individuals with significant injury or continuing progression of disease despite changes in the environment. Finally therapeutic measures for intervention might be better evaluated for their effectiveness in arresting cancer development as reflected by the glycosaminoglycan changes which in turn reflect changes in collagen formation.

SUMMARY AND HYPOTHESIS

Vinyl chloride appears to enter the body through the respiratory tract, the skin, or by swallowing; it is absorbed and transported to the liver by the systemic and portal circulatory systems. In the liver, the primary site of metabolism, it is oxidized by various enzyme systems: alcohol dehydrogenase at lower exposure levels; the peroxidase-catalase system at intermediate levels; and mixed function oxidases at higher levels. At the higher levels, the mixed function oxidase system transforms vinyl chloride to chlorooxiranes which are then spontaneously transformed into choroethanol and chloroacetaldehyde.

These two intermediate metabolites, chloroethanol and chloroacetaldehyde, are detoxified by conjugation with glutathione and cysteine-SH groups and are excreted in the urine. At even higher doses increasing amounts of the chloroacetaldehyde are further oxidized to chloroacetic acid and excreted as an end product in the urine. However, when chloroacetaldehyde and/or the chlorooxiranes exceed the detoxification threshold of the hepatocyte, this leads to hepatocellular toxicity and/or stimulation of the sinusoidal cells. This acute event in turn acts as a stimulating mechanism for increased collagen deposition in the space of Disse and sinusoidal areas as shown by electron and light microscopy studies. The increase in the collagen depostion in sinusoidal spaces then leads to disruption of hepatic cell surface function, disruption of the hepatic cords, coalition of the sinusoidal spaces and eventual peliosis hepatis. These lesions alternately lead to sufficient vascular dysfunction to add further to the biochemical manifestation of hepatic cellular injury.

Since it is highly unlikely that the unstable chlorooxiranes are able to be transported to adjacent cells and that the chloroacetaldehyde would most likely be conjugated or detoxified within the hepatocyte, an intermediate form, such as chloroethanol, which is transportable from the hepatocyte, may then move on to the adjacent sinusoidal lining cells, or possibly even further to other extrahepatic tissue. At these extrahepatic sites, an intermediate, such as chloroethanol, may then be converted to chloroacetaldehyde. The extrahepatic tissue sites are most likely unable to further convert the chloroacetaldehyde to chloroacetic acid nor to detoxify it sufficiently, if at all, by their own detoxification systems. This would allow a longer contact period with the cell's DNA. In addition many of the extrahepatic cells normally are regenerating at faster rates than hepatocytes, thus increasing the possibility of DNA derangement and ultimate carcinogenesis.

Alternatively, vinyl chloride itself may be taken up by extrahepatic tissue, oxidized but incompletely detoxified, allowing the cell itself to become susceptible to direct DNA injury. In this or similar manner, chemical metabolites may induce injury to the DNA in rapidly replicating cells at sites beyond the liver, thus accounting for other cancers developing with vinyl chloride.

The recent work by Maltoni's group, showing that exposure of newborn rats to the same dose of vinyl chloride as adult rats, results in primary hepatocellular carcinoma (40–45%) rather than in angiosarcoma (8–12%), lends further support to

the concept that the hepatocyte's ability to resist cancer transformation is dependent upon its ability to detoxify the mutagenic/carcinogenic metabolite of vinyl chloride.

This review of our present knowledge of vinyl chloride injury and cancer formation in man is, at best, a very rough hypothetical outline. With continued investigation and study it will allow us to more accurately and completely fill in the missing pieces of this fascinating puzzle, thus leading us to a better understanding of the pathogenesis of chemically induced cancer in the biologically complex human system.

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